

**ANTIGEN UPTAKE AND PRESENTATION
BY OVINE AFFERENT LYMPH DENDRITIC
CELLS**

Susan Nicola Coughlan

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UNIVERSITY OF EDINBURGH

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Name of Candidate Susan Nicola Coughlan

Address

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Dendritic cells (DC) are accessory cells distributed throughout non-lymphoid and lymphoid tissues, and also found in the blood and afferent lymph. These cells represent 1-10% of the leucocytes in ovine afferent lymph, and can be purified using pseudoafferent cannulation techniques. DC are essential as accessory cells for the initiation of primary immune responses, and are also the most effective antigen presenting cells for activation of T cells in the secondary response. However, the mechanisms underlying the potent accessory capability of DC are currently poorly understood. The aims of this thesis were to characterise DC surface markers and Fc receptor expression in primary and secondary immune responses, and to investigate the effect of enhanced antigen uptake via DC Fc receptors on T cell activation.

The phenotype of DC from the afferent lymph of sheep was investigated by flow cytometry. MHC class I, class II and CD1 were observed at high levels on the DC surface, while staining for immunoglobulins was variable. Cells obtained from the afferent lymph of primed sheep on antigen challenge *in vivo* showed pronounced and rapid modulation of surface markers, which may be required for processing and presentation of antigen and migration to the draining lymph node.

Further characterisation of DC demonstrated staining with polyclonal antisera to peptides of the bovine Fc receptor Type I for IgG. Western blot analysis of DC lysates identified bands of the correct sizes for the Type I and II Fc receptors for IgG, suggesting that both are expressed by ovine afferent lymph DC.

Functional studies were carried out to investigate the role of Fc receptors for IgG in the uptake and presentation of antigen by DC. Specific antibody greatly increased the response of CD4+ T cells to substimulatory concentrations of antigen presented by DC, depending on the antigen/ antibody ratio. F(ab')₂ portions of specific antibody and intact irrelevant antibody were completely ineffective. These results suggest enhanced presentation of antigen by DC following the uptake of immune complexes via Fc receptors. Similar results, although with a smaller degree of potentiation, were obtained using unfractionated peripheral blood mononuclear cells.

Fc receptors on DC would be important in secondary responses *in vivo*, in which specific antibody of IgG isotype is present. The potentiation of T cell activation may also play a role in diseases in which immune complexes are presented by DC.

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DECLARATION

The composition of this thesis and the experiments described are my own work, unless specifically stated in the acknowledgements section or text. No part of this work has been, or will be, submitted for any other degree, diploma or qualification.

Susan Nicola Coughlan

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ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen-presenting cell
BSA	bovine serum albumin
CD	Cluster of differentiation
CFA	complete Freund's adjuvant
Con A	concanavalin A
CSF	colony-stimulating factor
CTL	cytotoxic T cells
DC	dendritic cells
ED	extracellular domain
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorter
FCS	foetal calf serum
FcR	Fc receptor
FITC	fluorescein isothiocyanate
HSA	human serum albumin
IDC	interdigitating dendritic cell
Ig	immunoglobulin
IFN	interferon
IL	interleukin
ISCOM	immunostimulatory complex
LC	Langerhans cells
LMP	low molecular mass polypeptide
MAb	monoclonal antibody

MACS	magnetic activated cell sorter
MHC	major histocompatibility complex
mIg	membrane immunoglobulin
MLR	mixed leucocyte reaction
MI _s	minor lymphocyte stimulating antigen
NK	natural killer
NP-40	Nonidet P-40
OVA	ovalbumin
PBM _s	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PE	phycoerythrin
PMSF	polymethylsulphonyl fluoride
RBC	red blood cell
SDS-PAGE	sodium dodecyl sulphate polyacryamide-gel electrophoresis
SMAA	solid-matrix-antibody-antigen
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
WBC	white blood cell

CHAPTER ONE

INTRODUCTION

1. INTRODUCTION

1.1. Dendritic Cells

Dendritic cells (DC) are specialised accessory cells found in both lymphoid and non-lymphoid tissues throughout the body. They are very efficient in the generation of T-dependent immune responses, and are unique in their ability to activate resting T cells. In recent years much research has concentrated on their distinctive functional capabilities; however many key areas remain unclear.

DC have been identified in all non-lymphoid tissues with the exception of neural tissue. Langerhans cells (LC) of the epidermis are probably the best characterised of these interstitial dendritic cells (Schuler et al 1985). They are thought to act by capturing antigens in the periphery and migrating to the T cell areas of lymph nodes or spleen, via afferent lymphatics or the bloodstream. Here, DC interact with recirculating T cells, activating those specific for the antigen carried (Kripke et al 1990).

DC are essential for the generation of the primary immune response to antigen (Inaba et al 1990, Macatonia et al 1989). After the initial activation event, other antigen presenting cells (APCs) such as macrophages and B cells can further stimulate responding T cells for clonal expansion and effector function development (Metlay et al 1989). In spite of their potent accessory function, very little is known of the mechanisms by which DC accumulate antigen. This knowledge would facilitate the modulation of immune responses at a very early stage and may have implications for vaccine development, and possibly autoimmune diseases in which responses are mounted against self antigens.

DC from the afferent lymph of sheep form rosettes with IgG-coated erythrocytes, and take up antigen with greater efficiency in the presence of specific antibody, suggesting that immune complexes are attached to the DC surface via Fc receptors (FcR) for IgG (Harkiss et al 1990). IgM is detected on the surface of a proportion of lymph DC, and it is possible that this antibody could act as a concentrating mechanism for antigen in a

primary response (Bujdoso et al 1990). In this project, therefore, the mechanisms involved in the enhanced uptake of antigen by lymph DC, and the functional consequences of this enhancement in terms of the efficiency of antigen presentation were investigated.

1.2. Ontogeny: The DC Lineage

Although DC are known to arise from bone marrow progenitors, their development is not well characterised. DC are not detectable in fresh preparations of bone marrow but develop during short-term culture in serum-free medium (Steinman and Cohn 1974). DC precursors do not express major histocompatibility complex (MHC) class II molecules and are actively dividing in contrast to the functional progeny, which do not divide and express high levels of MHC class II (Bowers and Berkowitz 1986).

DC and monocytes have some functional and phenotypic properties in common, suggesting a common precursor (Austyn 1987). Tissue culture studies in human bone marrow preparations support this theory, with monocytes and DC identified within mixed colonies (Goordyal and Isaacson 1985, Reid et al 1990). A recent report by Inaba et al (1993) describes a common origin for murine granulocytes, macrophages and DCs. Colonies grown in semi-solid methylcellulose developed a small proportion of DC (1-2%) when cultures were supplemented with granulocyte/ macrophage colony-stimulating factor (GM-CSF), a cytokine known to have beneficial effects on DC viability *in vitro* (Witmer-Pack et al 1987, Macpherson 1989). DC, identified by phenotype and functional properties, were not seen when cultures were supplemented with the lineage-specific M-CSF or G-CSF.

Functional DC isolated from human blood express the myeloid markers CD13 and CD33 and the Fc receptors CD32 and CD64, although the latter are downregulated when DC are cultured. However, the monocyte/ macrophage marker CD14 is either absent or expressed at low levels by freshly isolated or cultured DC, and these cells are not capable of FcR-mediated phagocytosis (Thomas et al 1993a). Taken together with the superior accessory functions exhibited by DC, these factors suggest that DC and

monocytes share a common myeloid origin but differentiate along separate pathways giving rise to their distinctive features.

Proliferating DC precursors have also been identified in murine and human blood (Inaba et al 1992, Reid et al 1990). As in bone marrow cultures, supplementation with GM-CSF is required for their development. Studies with human CD34+ haematopoietic progenitor cells demonstrated the need for TNF- α in addition to GM-CSF for optimal production of DC *in vitro* (Caux et al 1992). Mature DC resembled Langerhans cells with the expression of CD1a and Birbeck granules.

It is possible that DC progenitors from the bone marrow enter the blood, and subsequently other tissues, and then proliferate extensively in these areas, undergoing maturation in response to the cytokines produced by other cell types. Indeed, it has recently been demonstrated that thymic DC develop from an intrathymic precursor population in common with T cells, and do not arrive in the thymus fully differentiated (Ardavin et al 1993). This also has implications for thymic selection (see below).

1.3. DC in The Induction of Immune Responses

1.3.1. Primary Responses

DC are extremely efficient accessory cells for T-dependent responses *in vivo* and *in vitro*. There seems to be an absolute requirement for DC in the development of a primary response where naive T cells must be activated (Austyn 1987, Metlay et al 1989, Ronchese & Hausmann 1993). DC were found to be necessary for initiation of the primary allogeneic mixed leucocyte reaction (MLR); B cells, B blasts and MHC class II-expressing peritoneal macrophages were ineffective (Inaba and Steinman 1984).

Priming of naive T cells *in situ* has been demonstrated in cattle and rodents. DC collected from the afferent lymph of non-immune cattle were pulsed *in vitro* with ovalbumin, and returned to the donor by intradermal injection after thorough washing.

Antigen-specific responses were detected in PBMs from 1-5 weeks after DC administration (McKeever et al 1992). In mice, lymphoid DC pulsed *in vitro* with protein or peptide antigens and injected into the footpad led to the development of antigen-specific CD4+ T cells in the draining lymph node (Inaba et al 1990, Levin et al 1993). Direct stimulation of host cells by antigen-bearing DC was shown by MHC restriction; pulsed DC of parental strain were administered to F1 hybrid recipients, and the T cells that were primed then responded to antigen in the context of the MHC class II products of the donor DC. Antigen-pulsed B cells and macrophages were unable to induce a primary response *in vivo*. DC obtained from thoracic duct cannulations of rats following mesenteric lymphadenectomy were effective at inducing T cell responses in naive animals, when antigen was acquired either *in vivo* or *in vitro*. (Liu & Macpherson 1993). In the latter experiment, as few as 500 antigen-pulsed DC were capable of sensitising specific T cells *in vivo*.

DC also act as APCs for CD8+ T cells in primary cytotoxic T lymphocyte (CTL) responses *in vitro* (Macatonia et al 1989). Nair et al recently reported that DC pulsed with antigen *in vivo* or *in vitro* could subsequently induce primary CTL responses *in vitro*, while macrophages were ineffective (Nair et al 1993). Others demonstrated the induction of CD8+ CTL by a single *in vivo* immunisation with peptide-pulsed DC (Takahashi et al 1993).

Induction of the primary antibody response *in vitro* requires DC as accessory cells. DC sensitise specific CD4+ T cells which can then stimulate antigen-carrying B cells (Inaba et al 1985). Syngeneic DC pulsed with a soluble protein antigen *in vitro* can elicit an antibody response *in vivo* when administered to naive mice, while pulsed B cells are ineffective (Sornasse et al 1992). However, antibody can only be detected following the injection of soluble antigen, at 12 days after DC administration, and therefore the antibody detected may represent a secondary response. Antigen-bearing DC probably sensitise specific CD4+ T cells, which can provide B cell help when soluble antigen is injected subsequently. This correlates with the findings of Liu & Macpherson (1993), where antigen-pulsed rat lymph DC did not produce a detectable antibody response on *in vivo* administration, but weak secondary antibody responses were evident after

boosting the animals with soluble antigen.

1.3.2. Secondary and Other Responses

DC are more efficient than macrophages or B cells in the generation of secondary immune responses (Steinman 1991). Fewer DC are required to elicit a measurable response due to their specialised antigen presenting functions, in several assay systems. Thus antigen-pulsed DC from the afferent lymph of cattle are 10-100 times more efficient than pulsed bovine monocytes in the stimulation of antigen specific T cells (McKeever et al 1991). DC are the most potent accessory cells for the allogeneic and syngeneic MLR and T cell responses to foreign protein antigens in humans (Van Voorhis et al 1982, Kuntz-Crow & Kunkl 1982, Van Voorhis et al 1983). In fact DC are the only accessory cells capable of eliciting the syngeneic MLR in murine systems, where T cells respond in the absence of exogenous antigen. Priming of mice with antigen before carrying out the assay does not affect the response, and it is suggested that a subpopulation of T cells is directly stimulated by DC; the mechanism may be important in self-recognition *in vivo* (Nussenzweig & Steinman 1986).

DC accessory activity has also been demonstrated in oxidative mitogenesis assays; here syngeneic T cells are pretreated with an oxidising agent such as sodium periodate then APCs are added in the absence of exogenous antigen. DC were found to be the major cell type capable of stimulating T cells in this system in rats (Klinkert et al 1982) and in humans, where DC elicited a response three times greater than that seen with macrophages or B cells as accessory cells (King & Katz 1989). In cloning studies, antigen-specific T cells could be cloned with 80% efficiency in the presence of only 1000 DC, while B cells and monocytes were <1% as effective (Langhoff & Steinman 1989).

1.4. Adaptations for Accessory Function

The unique ability of DC to initiate primary T-dependent responses has not been fully accounted for. However, contributing factors are likely to include the constitutive

expression of high levels of MHC products involved in antigen presentation, costimulatory/ adhesion molecules such as B7 (Young et al 1992), and possibly the production of cytokines with costimulatory functions such as IL-1 and IL-6 (Matsue et al 1992, Heufler et al 1992). Dendritic morphology and the decreased sialation of DC surface molecules allows increased contact with lymphocytes, while the migration patterns of DC from peripheral tissues to the T cell areas of lymphoid organs promote subsequent interaction with numerous potential responder cells. These features are covered in more detail below.

1.4.1. Surface molecules involved in accessory function

1.4.1.1. Antigen presentation

DC constitutively express MHC class II products, necessary for the presentation of peptide antigens to CD4+ T cells, at high levels. I-A and I-E are found in the mouse, while HLA-DP, DQ and DR are present on human DC (Steinman 1991). Ovine DC express MHC class II products equivalent to the human DQ and DR (Dutia et al 1993). Antigen challenge in a primed sheep causes an increase in expression of MHC class II molecules by afferent lymph DC. This correlates with enhanced ability of DC to present a second antigen to specific T cells (Hopkins et al 1989). Addition of a monoclonal antibody to MHC class II leads to total inhibition of T cell proliferation.

MHC class I molecules are also expressed at high levels by DC. These molecules are expressed by many cell types compared with MHC class II molecules. Expression of MHC class I products enables cells, including DC, to present endogenous antigens to CD8+ T cells for the induction of CTL (Townsend et al 1986).

LC and DC from ovine afferent lymph also express CD1 (Bujdoso et al 1989), which is similar in structure to MHC class I molecules and is associated with β_2 microglobulin. In humans, five genes for CD1 have been identified and termed CD1a-e (Calabi et al 1991). Different cell types may express the product of one or more CD1 genes. Two genes for CD1 have been located in the mouse (Bradbury et al 1988).

In sheep at least four genes have been identified (Ferguson 1993). It has been suggested that the CD1 antigens form a family of non-classical MHC class I molecules (Van de Rijn et al 1983). However, unlike MHC class I, CD1 does not show significant polymorphism (Calabi et al 1991). CD1 molecules are thought to function in the presentation of antigens to T cells bearing $\gamma\delta$ TCRs (Porcelli et al 1989, Haas et al 1993). CD1b has also been reported to restrict the response of human CD4-, CD8- T cells expressing the $\alpha\beta$ TCR to a microbial antigen (Porcelli et al 1992). DC in afferent lymph and epidermal LC are the major sites of expression of CD1 in the periphery, and they may be involved in the presentation of antigen to the large numbers of $\gamma\delta$ T cells also present in afferent lymph (Mackay et al 1988a) or the epidermis (Kuziel et al 1987).

Other “non-classical” class I molecules, known as the class Ib molecules, may also play a role in antigen presentation (Germain & Margulies 1993). The MHC class Ib genes map to the MHC area, but their products are distinct from those of class I genes in that they show limited polymorphism and restricted tissue expression, although in common with class I molecules they are associated with β_2 microglobulin. CD1 is not included in the MHC class Ib category, as its genes do not map to the MHC region (Calabi et al 1991). Various murine $\gamma\delta$ T cell subsets recognise antigens presented by the products of genes in the TL or H-2T regions, although the physiological relevance of this recognition is at present unclear (Bluestone et al 1988, Van Kaer et al 1991). One report describes TL products on the surface of murine LC (Rowolen et al 1983). The class Ib molecules have been proposed as a first line of defence against microbial infections of epithelial linings, due to their localisation in these tissues and their association with a restricted peptide repertoire (Sperling & Bluestone 1993).

Thus DC express all the presentational molecules required for interaction with both $\alpha\beta$ and $\gamma\delta$ T cells constitutively. In contrast, monocytes/ macrophages express variable amounts of MHC class II products, and must be activated before optimal levels of class II appear on the cell surface (Unanue 1984).

1.4.1.2. Adhesion: cluster formation

Most APC types can form clusters with T cells in an antigen-dependent manner; the unique feature of DC is their ability to cluster resting T cells non-specifically. The initial event is reversible enabling one DC to interact temporarily with many T cells to locate the small number of those responsive to the antigen carried (Inaba & Steinman 1986). Cluster formation is promoted by the dendritic morphology of DC; numerous processes increase surface area and motile lamellapodia or 'veils' extend and retract to contact neighbouring cells (Steinman 1991). DC surface molecules have a lower level of sialation than other cells (Boog et al 1989), allowing a closer apposition between cell surfaces due to reduced negative charge. The decreased degree of sialation is due to biosynthesis, and is not the result of neuraminidase action at the DC surface. T cells specific for peptides on the DC surface remain in the clusters and are activated, while others are released.

Several molecules on the DC surface interact with ligands on responding T cells to mediate adhesion: LFA-3 (CD58) with CD2, ICAM-1 (CD54) with LFA-1 (CD11a/CD18) and B7 with CD28 or CTLA-4 (Makgoba et al 1989, Young et al 1992). LFA-1, LFA-3 and CD2 are all expressed at greater levels on memory compared with naive T cells, increasing their ability to interact with DC bearing ligands for these molecules (Springer 1990). LFA-1 also undergoes a change from a low avidity to a high avidity state after crosslinking of the TCR or signalling via CD2, thus promoting intercellular adhesion. The increase in affinity correlates with protein kinase C-dependent phosphorylation of serine residues in the β subunit (CD18) of LFA-1 (Valmu et al 1991), and occurs rapidly, with peak avidity between five and ten minutes after TCR stimulation and a return to the low avidity state by thirty minutes (Springer 1990). The avidity of LFA-1 on the DC surface for ICAM-1 on the T cell may increase in a similar manner.

Cluster formation with resting T cells is an active process, requiring a temperature of 37°C, an intact DC cytoskeleton and intracellular protein kinase activity (Scheeren et al 1991). Bidirectional interaction between LFA-1 and ICAM-1 seems to be the central

event in binding; monoclonal antibodies (mAbs) to these surface molecules inhibit cluster formation by 70%. However, the initial antigen-independent interaction is not affected and the mAbs act by decreasing the stability of clusters after formation (Inaba & Steinman 1987). Other ligand pairs must also be involved as binding is not completely inhibited by mAbs specific for LFA-1 and ICAM-1. King and Katz demonstrated a role for LFA-3/ CD2 adhesion molecules in the association of human tonsillar DC with T cells (King & Katz 1989), but this was not confirmed in studies involving blood DC (Scheeren et al 1991). Sheep afferent lymph DC express CD2 and LFA-3 (Bujdoso et al 1990), therefore interaction of these molecules may occur to facilitate homoadhesion between DC and T cells.

1.4.1.3. Costimulation

Contact of the TCR with antigen in the context of MHC products provides a signal to the T cell but this is insufficient for its activation. Indeed, the absence of a second, costimulatory signal provided either by a cytokine or surface molecule on the presenting cell may lead to a state of anergy, preventing subsequent activation of the T cell (Schwartz 1990). B7 is an important costimulatory molecule expressed by DC. Young et al (1992) reported the constitutive expression of B7 by human blood DC, but a more recent study suggests that B7 is expressed only after activation of DC, and is present on IDC from lymphoid tissues but not on blood DC or LC (Hart et al 1993). B7 is also found on activated B cells and monocytes (Dustin & Springer 1989, Freeman et al 1987). MABs to the T cell ligands for B7, CD28 and CTLA-4, inhibited proliferation in the allogeneic MLR. MABs against T cell molecules CD2, CD4 and CD11a also decreased proliferation in this assay (Young et al 1992). When added together the blocking effect was synergistic and near total inhibition was achieved, indicating that all these molecules are involved in T cell activation. The expression of costimulatory molecules is also increased after antigen-specific interactions; B7 and ICAM-1 on the DC surface are both upregulated, while the avidity of T cell LFA-1 is increased after signalling through CD2 or CD3 as described above. Other DC accessory molecules may also be important in T cell activation; for example the murine heat-stable antigen provides a significant costimulatory signal from murine DC (Liu et al 1992). The

superior accessory function of DC may be partly explained by the high constitutive or induced expression of costimulatory molecules compared with other APCs, enabling DC to activate resting T lymphocytes. However, once sensitised, T cells may be stimulated to respond to antigen by other APC types (Austyn 1987).

1.5. Cytokine production

Certain cytokines such as IL-1 and IL-6 have costimulatory properties for T cells (Dinarello 1992), and in the past, macrophage release of IL-1 was considered essential for the activation of T cells in the presence of specific antigen (Steinman 1991). The production of cytokines by DC has not been comprehensively investigated, although LC produce IL-1 and IL-6 (Matsue et al 1992, Enk & Katz 1992), and the expression of IL-1 β mRNA is upregulated on culture of these cells. The release of IL-1 β by murine splenic DC has also been reported (Heufler et al. 1992). IL-1 β produced by epidermal LC is essential for the induction of primary immune responses in the skin (Enk et al. 1993). The accessory function of DC is enhanced in the presence of IL-1, suggesting that DC must express receptors for IL-1 (Koide et al. 1987). The presence of these receptors has been demonstrated by the binding of radiolabelled IL-1 to DC from ovine afferent lymph (Fiskerstrand 1994).

In one study, DC and responding T cells were separated from T cells stimulated with anti-CD3 mAbs by a 0.22 μ m filter. The latter cells should proliferate when a second signal is supplied, as the TCR is already occupied by anti-CD3 mAbs. IL-2 was produced and crossed the filter but the T cells did not respond, indicating that a surface-bound molecule or molecules were necessary for activation (Inaba et al 1989).

A recent study demonstrates the effects of IL-10 on immune responses initiated by DC. DC-induced production of gamma interferon (IFN- γ) by both Th1 clones and bulk populations of CD4 $^{+}$ and CD8 $^{+}$ T cells was inhibited on the inclusion of IL-10 in *in vitro* cultures, while proliferation of the responding T cells was not affected (Macatonia et al 1993). This suggests a role for IL-10 in the inhibition of cell-mediated responses, and demonstrates separate regulation of proliferation and cytokine production in the

responding cells.

1.6. Antigen uptake

The methods employed by DC in the accumulation of antigens are not fully understood. DC isolated from murine spleen are poorly phagocytic (Steinman 1991), while DC in the afferent lymph of sheep can take up immune complexes (Hall & Robertson 1984). FcR for IgG are found on murine and human Langerhans cells, and DC from murine pulmonary tissue and human afferent lymph (Schuler et al 1985, Spry et al 1980, Astier et al 1994), while those DC obtained from murine lymph nodes or spleen, or human peripheral blood, do not express these molecules (Van Voorhis et al 1982). However, the method of isolation employed may also be important, with more recent reports showing low levels of FcR on the surface of DC obtained from murine spleen by a rapid isolation technique (Crowley et al 1990), and on DC from human blood (Thomas et al 1993a). Complement receptors are also present on DC; CD11b is detected on >80% of human LC, but only a trace population of DC from lymphoid tissues and afferent lymph, while CD11c is expressed by both DC and LC (Steinman 1991, Nestlé et al 1993). DC generally lack mannose-fucose receptors (Pugh et al 1983), although freshly isolated LC from some strains of mice do express these receptors, which are downregulated on culture (Reis e Sousa et al 1993). Other receptors such as those for lipoproteins have not been identified.

Although the mechanism of antigen uptake is not clear, it is certain that DC do acquire antigens *in vivo* and *in vitro*. Sheep afferent lymph DC draining the site of intradermal antigen administration can activate specific T cells *in vitro* (Bujdoso et al 1989). A similar effect has been demonstrated with pulmonary DC after application of antigen in aerosol form (Holt et al 1987), and with DC in afferent lymph draining the intestine after oral administration of antigen to rats (Liu & Macpherson 1991).

In a primary immune response, DC must acquire sufficient antigen for the activation of resting T lymphocytes in the absence of specific antibodies. DC in the afferent lymph of sheep bear surface immunoglobulins at variable levels (Miller & Adams 1977). Surface

IgM, which is likely to be cytophilic, has been proposed as a concentrating mechanism for antigen in a primary response (Bujdoso et al 1990). IgM antibodies may form complexes with antigen and attach to the DC membrane via complement receptors or receptors for IgM, facilitating the uptake of trace amounts of antigen. Alternatively, non-specific pinocytosis may play a major role in the accumulation of antigen by these cells in a primary response; DC are highly efficient in fluid-phase pinocytosis (Barfoot et al 1989, Drexhage et al 1979). DC isolated from murine lymphoid tissues were initially thought to have poor endocytic activity (Kapsenberg et al 1986, Inaba et al 1990). However, DC from murine spleen have recently been shown to be as endocytically active as other APC types (Levine & Chain 1992). Endosomal traffic was assessed by measurement of the accumulation and subsequent exocytosis of fluid-phase markers in a flow cytometric assay. While these DC had a less active early endosomal compartment than activated B cells or macrophages, the late endosomal compartment, proposed as the site of antigen processing and association with MHC class II molecules (Peters et al 1991) was equivalent in size and activity. A recent study demonstrated the phagocytosis of mycobacteria by proliferating DC precursors from murine bone marrow cultures, showing that these DC can also internalise particulate antigens for subsequent presentation (Inaba et al 1993). Microorganisms such as yeast and bacteria were phagocytosed by freshly isolated LC, although this capacity was downregulated on culture *in vitro* (Reis e Sousa et al 1993).

Afferent lymph DC of sheep form rosettes with antibody-coated erythrocytes, and take up antigen far more efficiently in the presence of specific antibodies of the IgG isotype (Harkiss et al 1990). This enhanced uptake requires an intact Fc portion of antibody, suggesting the involvement of Fc receptors for IgG on the DC surface. Such a mechanism would be important in secondary responses *in vivo*, where specific antibody is available, and in disease conditions where immune complexes are present, especially if the increased uptake of antigen via FcR facilitated T cell activation.

1.7. DC Migration and Maturation

1.7.1. Langerhans Cells

Although DC are present in most non-lymphoid tissues, the LC of the epidermis are the best characterised. LC are thought to be the precursors of interdigitating DC (IDC) in the lymph node, travelling via afferent lymph as veiled cells (Knight et al 1982). LC have been identified in lymphatics draining the epidermis after application of contact sensitising chemicals, and subsequently in the draining lymph node (Silberberg-Sinakin et al 1976). LC migration from the epidermis to lymphatics in allografts and isografts also occurs (Larsen et al 1990). In this study, LC migrated from epidermal cultures into the surrounding medium, implying the involvement of local inflammatory stimuli in migration. TNF- α has been proposed as a specific stimulus for the initiation of LC migration *in vivo* (Cumberbatch & Kimber 1992).

DC bearing high levels of antigen can be isolated from the draining lymph node four hours after skin-painting with fluorescein isothiocyanate (Macatonia et al 1987). These cells carry antigen in immunogenic form and can elicit T cell responses *in vitro* and *in vivo* (Moll et al 1993). Once present in the lymph node, DC do not emerge in the blood or efferent lymph and probably die in the node (Fossum 1988).

Further evidence that at least a proportion of IDC are derived from epidermal LC is provided by the phenotypic and functional maturation of LC which occurs during culture and is thought to parallel the *in vivo* process (Puré et al 1990). Freshly isolated LC are capable of processing and presenting native protein antigens, but cannot sensitise resting T cells (Romani et al. 1989). After an *in vitro* culture period, immunostimulatory capacity is increased significantly but LC become incapable of presenting native antigens (Streilein & Grammer 1989). Phenotypic changes include increases in the expression of MHC class II and class I, expression of the low affinity receptor for IL-2, and downregulation of Fc γ RII (Shimada et al. 1987, Witmer-Pack et al. 1988). Changes in culture are dependent on the presence of GM-CSF, and may be increased by IL-1 (Witmer-Pack et al. 1987, Heufler et al. 1988). Cultured LC thus

resemble DC isolated from lymphoid tissues in both phenotype and function.

The decline of antigen processing in cultured LC is not due to decreased uptake of antigen; fresh and cultured LC accumulate a comparable amount of the pinocytic tracer rhodamine-ovalbumin in perinuclear granules (Puré et al. 1990). MHC class II expression is increased on culture, and cells can efficiently present peptides, which do not require processing. However, the biosynthesis of MHC class II molecules decreases dramatically on culture, with no synthesis detectable by 20 hours of culture (Puré et al. 1990). Production of the invariant chain cannot be detected at this stage either. This suggests that newly formed MHC class II molecules are required for interaction with peptides in LC; in support of this, antigen processing in freshly isolated LC is blocked by cyclohexamide which interferes with the synthesis of MHC class II molecules. This correlates with the finding that nascent MHC class II molecules are required for association with antigenic peptides in other APC types (Neefjes et al 1990, Davidson et al 1991, Lanzavecchia et al 1992).

The disappearance of certain intracellular organelles of acidic pH also coincides with the loss of processing ability by LC (Stossel et al 1990). These are largely late endosomes and Birbeck granules and may be required for antigen cleavage and peptide production. Although the exact site of peptide production and association with MHC class II molecules has not been defined, the presence of an acidic compartment is an essential requirement (Jensen 1990).

Once fresh LC have taken up an antigen they can retain it for up to two days in culture (Puré et al 1990). This may be necessary *in vivo* to ensure that antigens captured in the peripheral tissues are not displaced before they can be presented to specific T cells. Indeed, the reciprocal antigen processing ability and immunostimulatory capacity of fresh and cultured LC probably helps to ensure that antigen is presented in immunogenic form at the site where interaction with specific T cells is most likely.

1.7.2. The Role of DC in Antigen Transport in the Lymph Node

Follicular dendritic cells (FDC) in the lymph node retain antigen for long periods on their cell membrane and are thought to be important in the maintenance of B cell memory and the secondary antibody response (Gray & Skarvall 1988, Tew et al 1989). In a secondary immune response, immune complexes in the afferent lymph may be transported from the subcapsular sinus to the germinal centre by cells with dendritic morphology; antigen is then transferred to FDC for interaction with B cells (Szakal et al 1989). The exact nature of these antigen transport cells is not known, but they may be part of the DC lineage. FDC release antigen in the form of iccosomes, immune complex-coated bodies which are subsequently taken up by B cells and stimulate the production of antibody.

1.7.3. Transplantation

Because of their immunostimulatory ability and migratory properties, DC have been proposed as important passenger leucocytes involved in the rejection of transplanted tissues (Austyn & Larsen 1990). DC would act by sensitising host T cells to graft antigens, thus initiating rejection. Depletion of DC from pancreatic islet grafts in mice has proved effective in preventing rejection by allogeneic recipients (Faustman et al 1984). It is not known whether sensitisation to the graft occurs peripherally or in lymphoid organs. One study of skin grafts in guinea pigs demonstrated a requirement for intact lymphatic drainage for rejection to occur, suggesting that DC from the grafts migrated to draining lymph nodes and initiated rejection centrally (Barker & Billingham 1968). However, Tilney & Gowans, using a similar model in rats, found that rejection did occur but was delayed. They suggested that sensitisation of host T cells was taking place in the graft itself (Tilney & Gowans 1970).

More recent studies have investigated fully vascularised organ grafts and the migration of DC via blood. Radiolabelled DC injected intravenously migrated only into the spleen and liver, with the former the major site of radioactivity (Kupiec-Weglinski et al 1988). In a rat cardiac allograft model, DC of donor origin were detected in the host spleen

soon after transplantation. Appearance of donor DC in the spleen corresponded with sensitisation to the graft (Larsen et al 1990). Further experiments showed that host DC did not migrate into cardiac or skin allografts (Austyn & Larsen 1990). The modification of grafting procedures involving depletion of donor DC or measures preventing their migration to host lymphoid tissues could be a factor in the improvement of graft survival.

1.8. Thymic DC and Tolerance

Selection of thymocytes bearing self MHC and the induction of tolerance to self antigens is thought to occur in the thymus. DC are present in the thymus at the corticomedullary junction where intermediate T cell stages between immature double-positive and mature single-positive cells reside, and in the medulla (Inaba et al 1991). Two populations of thymic DC were identified (Inaba et al 1988). One population expressed MHC class II and represented 0.1-0.3% of thymus cells. These DC were effective as accessory cells in thymocyte mitogenesis assays, with their stimulatory function increasing on pre-incubation with IL-1. MHC class II-negative DC developed class II expression and corresponding stimulatory activity when cultured in the presence of IL-1, indicating that these class II-negative DC are precursors of the mature, functional thymic DC.

A common precursor population in mouse thymus for both T cells and DC has been reported (Ardavin et al 1993). This precursor did not express MHC class II, and DC did not develop from later stages of the T cell lineage after gene rearrangement. As thymic DC develop from an intrathymic precursor, newly formed T cells are likely to interact with DC expressing only self antigens rather than DC having acquired exogenous antigens before entering the thymus. Other mechanisms would be required to generate tolerance to self antigens not present within the thymus.

The role of DC in tolerance induction was investigated by injection of minor lymphocyte stimulating antigen (Mls)-incompatible APCs into the thymi of neonatal mice (Inaba et al 1991). Responses were assessed by the demonstration of graft-

versus-host-response (GVHR) following injection of thymocytes into the footpads of Mls-incompatible recipients. Differences in the mode of tolerance induction by distinct APC types were observed. Thymic B cells caused deletion of reactive thymocytes while DC induced anergy; specific thymocytes were not deleted but GVHR did not occur. Macrophages were completely ineffective and tolerance did not occur.

In a fetal thymus organ culture system, again with Mls as antigen, cooperation between DC and Mls-expressing B cells was required for elimination of reactive T cell clones (Mazda et al 1991). Thus B cells in this *in vitro* system could not tolerate T cells alone, but probably passed Mls antigen to DC which in turn induced tolerance. This contrasts with the previous *in vivo* work; however in the latter case it is possible that Mls-incompatible B cells could transfer antigen to host DC after injection into the thymus, with the latter cells then inducing tolerance.

1.9. Pathways of Antigen Processing and Presentation

Intracellular and extracellular antigens are generally processed and presented by distinct pathways. Endogenous antigens produced in the cell cytoplasm, eg by viruses, are usually presented to CD8+ T cells in association with class I MHC molecules. Many cell types express class I products, and can potentially act as targets for CTL activity. In contrast only a few specialised cell types carry the MHC class II molecules required for the presentation of exogenous antigens to CD4+ T cells. These T cells are then activated to provide B cell help in the production of antibodies specific for the extracellular antigen. DC carry both MHC products and can therefore present intracellular and extracellular antigens to CD8+ and CD4+ T cells respectively.

In mice, CD4+ "helper" T cells have been further divided into two subsets depending on their cytokine profiles (Mosmann & Coffman 1989). Th1 cells secrete IL-2 and IFN- γ on activation and thus promote macrophage activation and cell-mediated responses such as delayed-type hypersensitivity (DTH), while Th2 cells produce IL-4, IL-5 and IL-10, and stimulate the production of antibody including IgE by B cells. The development of mast cells and eosinophils is also promoted by the Th2 subset. One Th

subset characteristically dominates in the immune response to a particular antigen, and each subset is able to suppress development of the other by the production of cytokines. Thus the IFN- γ produced by Th1 cells inhibits proliferation of Th2 cells, while Th2 cells in turn produce IL-10 which inhibits the production of IFN- γ by the Th1 subset (Fiorentino et al 1989). In some diseases, the dominant subset determines the outcome of the condition. In murine leishmaniasis, for example, a Th1 response with the production of IFN- γ and induction of cell-mediated responses leads to an effective immunity and healing of a localised lesion in resistant strains. In contrast, a Th2 response characterised by IL-4 and antibody production occurs in susceptible Balb/c mice, resulting in disseminated and often fatal disease (Heinzel et al 1989). Th1 and Th2 cells are thought to develop from a common precursor after TCR-mediated activation (Coffman et al 1991). The properties of different APCs, cytokine production by these or other cells, the amount of antigen available and epitope specificity have all been proposed as factors potentially responsible for the differentiation of uncommitted precursor cells into Th1 or Th2 types (Coffman et al 1991, Wang et al 1993, Schmitz et al 1993).

1.9.1. Processing of Endogenous Antigens

To be presented on MHC class I molecules for presentation to CD8⁺ T cells, antigens must be located within the cytoplasm of the presenting cell. Peptides are produced by protease cleavage of cytoplasmic proteins. The low molecular mass polypeptide (LMP), a large proteolytic complex related to the proteasome, is thought to be capable of producing multiple peptides simultaneously from a protein substrate. Peptides are then delivered into the endoplasmic reticulum (ER) by a transporter for association with class I molecules. The peptide transporter is thought to be the product of the genes *Tap-1* and *Tap-2*, which, with genes encoding the LMP, are found in the class II region of the MHC (Trowsdale et al 1990).

Once inside the ER, peptides bind to newly synthesised class I molecules consisting of a glycosylated heavy chain with three extracellular domains, non-covalently associated with β 2 microglobulin (Bjorkman et al 1987). A conformational change is induced on

peptide binding, resulting in stabilisation of the molecule's structure. MHC class I molecules without bound peptides dissociate rapidly at physiological temperatures. The antigen-binding groove of class I is formed by the association of the 1st and 2nd domains of the heavy chain, while the membrane proximal 3rd domain interacts with CD8 (Germain & Margulies 1993). The antigen groove accommodates a peptide of nine amino acids (Schumacher et al 1991), and there is some evidence that peptides of this size are preferentially produced by the LMP (Brown et al 1991). The groove incorporates a set of pockets extending between the floor and walls of the binding domain (Garrett et al 1989). MHC class I molecules are highly polymorphic (Klein 1986), and this is evident in the differing shape and character of the pockets in the binding groove, determining which peptides can be accommodated in the binding region (Bjorkman et al 1987). Allele-specific motifs have been defined in peptides eluted from MHC class I molecules of different haplotype, with amino acids commonly found at a particular position being classified as anchor residues (Falk et al 1991). For example, peptides eluted from HLA-B27 class I molecules were sequenced and the motif of an arginine at position 2 and a basic residue at position 9 was identified (Jardetzky et al 1991). Other work has demonstrated the importance of hydrogen bond formation between the NH₂-terminal amine of the peptide and conserved MHC residues such as the tyrosines at positions 7, 59 and 171. The terminal carboxyl group, and the first and penultimate amino acids of the peptide also form hydrogen bonds with other conserved class I residues (Matsamura et al 1992, Zhang et al 1992). These conserved bonds help to explain how polymorphic class I molecules can each bind a large number of different peptide sequences (Germain & Margulies 1993).

Class I MHC molecules with bound peptides are exported to the cell surface via the Golgi; during this process the pathway can be blocked by Brefeldin A, which prevents movement of membrane proteins from the ER to the Golgi. This inhibitor of class I presentation has no effect on the class II processing pathway (Nuchtern et al 1989).

1.9.2. Processing of Exogenous Antigens

Extracellular antigens enter endocytic vesicles of acid pH after internalisation. Antigens

pass through early and late endosomes to lysosomes, and are at some point along this pathway are cleaved by protease action to produce peptides. Treatment with lysosomotropic agents such as chloroquine and ammonium chloride neutralises these intracellular compartments and blocks the MHC class II processing pathway (Ziegler & Unanue 1982), while the endogenous class I pathway is unaffected. It is uncertain how the neutralisation of these acidic compartments blocks processing in the exogenous pathway; the degradation of antigens to peptide form may be inhibited, or alternatively dissociation of the invariant chain from the class II molecules to allow peptide binding may fail to occur (Germain & Margulies 1993).

Class II molecules are assembled in the ER. Molecules consist of a non-covalently associated α and β chain, each with two extracellular domains. The detailed structure of the human MHC class II molecule HLA-DR1 has recently been determined, and is similar to that of class I (Brown et al 1993). The $\alpha\beta$ heterodimers appear to associate as dimers of dimers. This suggests that class II molecules at the cell surface are able to form dimers at some stage, possibly during recognition by CD4 T cells. The resulting structure could lead to an increased affinity for CD4, and cross-linkage of TCRs may be important in stimulatory signalling in the responding T cells. There are also detailed changes in the structure of the peptide binding region, which is formed by the interaction of the $\alpha 1$ and $\beta 1$ domains. The conserved residues in the class I peptide groove which bind the peptide NH_2 terminus and the C terminus are not found in class II sequences (Brown et al 1993). The ends of the class II site are thus more open than those of class I, and bound peptides are longer and more varied in size, projecting out of the groove at each end. Peptides eluted from MHC class II molecules vary from twelve to more than twenty amino acids in length (Rudensky et al 1991, Chicz et al 1992). Amino acid motifs for binding to particular class II molecules have been identified, and these usually involve only two or three residues. These motif residues are thought to occupy polymorphic pockets in the class II binding region, of a similar type to those found in the class I binding groove (Germain & Margulies 1993). A distinct pocket in the binding site of HLA-DR1 has been identified, which accommodates a side chain of the bound peptide (Brown et al 1993). The site of association between MHC class II molecules and CD4 has been determined as the $\beta 2$

domain, which is structurally analogous to the CD8-binding region in the $\alpha 3$ domain of MHC class I molecules (Konig et al 1992, Cammarota et al 1992). If class II molecules do exist in dimer form during interactions with T cells, the structure determined by Brown et al suggests that CD4 might also contact a region of the $\alpha 2$ domain of one of the class II molecules.

A third component, the invariant chain, associates with the α and β chains before export of the class II molecule from the ER. The invariant chain has been proposed to target class II molecules to the endocytic route and also to prevent the binding of self peptides before class II molecules encounter exogenous peptides in endocytic vesicles. On arrival in an endosomal compartment, the invariant chain is degraded (Germain & Margulies 1993) to allow peptide binding to occur. MHC class II molecules are expressed on the cell surface in mutants lacking the invariant chain, but expression is impaired to a variable extent (Schaiff et al 1991). The invariant chain also seems to have a role in the correct folding and conformation of the $\alpha\beta$ dimers (Anderson & Miller 1992), and the association of certain peptides with MHC class II dimers is dependent on the prior association of the dimers with the invariant chain (Peterson & Miller 1990). Binding of peptide to MHC class II molecules causes a conformational change leading to increased stability of the class II dimer (Sadegh-Nasseri & Germain 1991). This is similar to the situation with class I MHC molecules, where peptide association with the heavy and β_2 microglobulin chains gives stability of the complex.

The exact site of association of MHC class II molecules with peptide is uncertain. Processed antigen can be detected on the surface of APCs by specific T cells as little as 20 minutes after endocytosis, and in some studies with human and mouse APCs the late endosomal compartment has been implicated as the site of association (Davidson et al 1991, Guagliardi et al 1990). Others have suggested the involvement of lysosomes (Pieters et al 1991, Harding & Geuze 1993).

A role for intracellular chaperone proteins in the transport of peptides and their association with MHC class II molecules has been proposed. Pierce and colleagues

suggested that molecules of the heat shock protein (hsp) 70 family, which possess peptide-binding capacity, could scavenge partially degraded proteins from lysosomal compartments and recycle them to the endosomes for binding to class II molecules at this site (Pierce et al 1991). Homology does exist between the peptide-binding regions of hsp70 and MHC class II molecules (Flajnik et al 1991), but there is no firm evidence to support this transfer theory at present (Germain & Margulies 1993).

After peptide binding has occurred, MHC class II molecules are transported to the cell surface, allowing interactions with responder cells specific for the antigen presented. Recycling of class II molecules from the cell surface to an endosomal compartment has been demonstrated (Salamero et al 1990, Reid & Watts 1990). However, this recycling may be of limited functional importance, as only newly synthesised molecules seem to associate with peptides derived from exogenous antigen (Davidson et al 1991, Lanzavecchia et al 1992). MHC class II molecules can also present exogenously added peptides without processing, if these are used at high enough concentrations. Added peptides may displace those already present on MHC class II molecules, or alternatively they may enter the antigen grooves of "empty" MHC class II molecules present on the cell surface (Germain & Hendrix 1991).

1.9.3. Endogenous Antigens Presented By Class II Molecules

Although antigens produced endogenously are usually presented by class I molecules, and exogenous antigens taken up by the cell follow the class II presentation pathway, there are exceptions to this general rule. Exogenous antigens can be presented to CD8+ T cells on class I molecules (Rock et al 1990, Reddy et al 1991). This requires penetration from the endocytic system to the cytoplasm, for example by osmotic shock in *in vitro* studies (Moore et al 1988). However, in some cases, exogenous antigens with no known method for entering the cytoplasm can elicit CD8 T cell responses (Carbone & Bevan 1990, Collins et al 1992). Similarly, intracellular pathogens such as *Leishmania* and *Salmonella* which reside in vacuoles and have no obvious access to the cytoplasm, can still elicit class I-restricted T cell responses (Flynn et al 1990, Farrell et al 1989). A recent report describes a novel class I processing pathway employed for

the processing and presentation of phagocytosed bacteria. In this study, presentation by class I molecules was not inhibited by either Brefeldin A or cyclohexamide, which block the recognised class I pathway (Pfeifer et al 1993).

There is also evidence for the presentation of endogenously synthesised antigens by class II molecules, particularly for cell surface or secreted proteins which can enter the endosomal processing pathway (Weiss & Bogen 1989, Bikoff & Eckhardt 1989). However, proteins that are produced in the cell cytoplasm and are not membrane-associated or secreted can also be presented by MHC class II molecules, as can some proteins which are retained in the ER and do not enter the cytoplasm in detectable quantities (Brooks et al 1991). The mechanisms involved in each case are unclear, however it is possible that a small amount of a protein with an ER retention signal could leave the ER and enter the endosomal pathway, while cytoplasmic proteins are more likely to be taken up into vacuolar compartments by autophagy, for processing in endosomes or lysosomes (Brooks & McCluskey 1993). The pathway involved in the presentation of endogenously synthesised antigens by class II molecules is inhibited by chloroquine but not by Brefeldin A, indicating that it is separate from the class I pathway for the processing of endogenous antigens (Jaraquemada et al 1990).

Antigens incorporated in immunostimulatory complexes (ISCOMS) may be presented by both class I and class II molecules, potentially activating CD8 and CD4 T cells, resulting in CTL and humoral responses against the given antigen (Takahashi et al 1990). A similar picture of T cell activation is seen following the administration of antigen in the form of solid-matrix-antibody-antigen (SMAA) complexes (Randall & Young 1991). This has implications in the development of vaccines to certain pathogens where protective immunity is dependent on such a dual response.

1.10. Fc Receptors for IgG

The expression of Fc receptors for IgG by APCs such as macrophages and DC enables them to take up antigen with increased efficiency in the presence of specific antibody, as occurs in secondary immune responses *in vivo*. Enhanced presentation of antigen

following its uptake via FcR has been demonstrated for different APC types (Section 1.11.1, below).

1.10.1. Structure

Fc receptors (FcR) for IgG have recently been the object of much investigation. There are three types of Fc γ R; Fc γ RI (CD64) has high affinity for monomeric IgG and is saturated with immunoglobulin (Ig) under physiological conditions, while Fc γ RII (CD32) and Fc γ RIII (CD16) bind only complexed IgG in detectable quantities (Ravetch & Kinet 1991). All are members of the immunoglobulin superfamily (Williams & Barclay 1988) and consist of a variable number of Ig-like extracellular domains, a transmembrane section and cytoplasmic portion. Fc γ RI has three extracellular domains while Fc γ RII and Fc γ RIII have only two (Fig.1). The first two domains of Fc γ RI are structurally and functionally related to those of the low affinity receptors; amino acid identity between murine Fc γ RI and Fc γ RII being 40% in this region (Hulett et al 1991). It is thought that the third domain of Fc γ RI confers its unique affinity for monomeric IgG (Ravetch & Kinet 1991). The cytoplasmic domain is required for localisation of receptor and ligand into clathrin-coated pits and subsequent internalisation (Miettinen et al 1991).

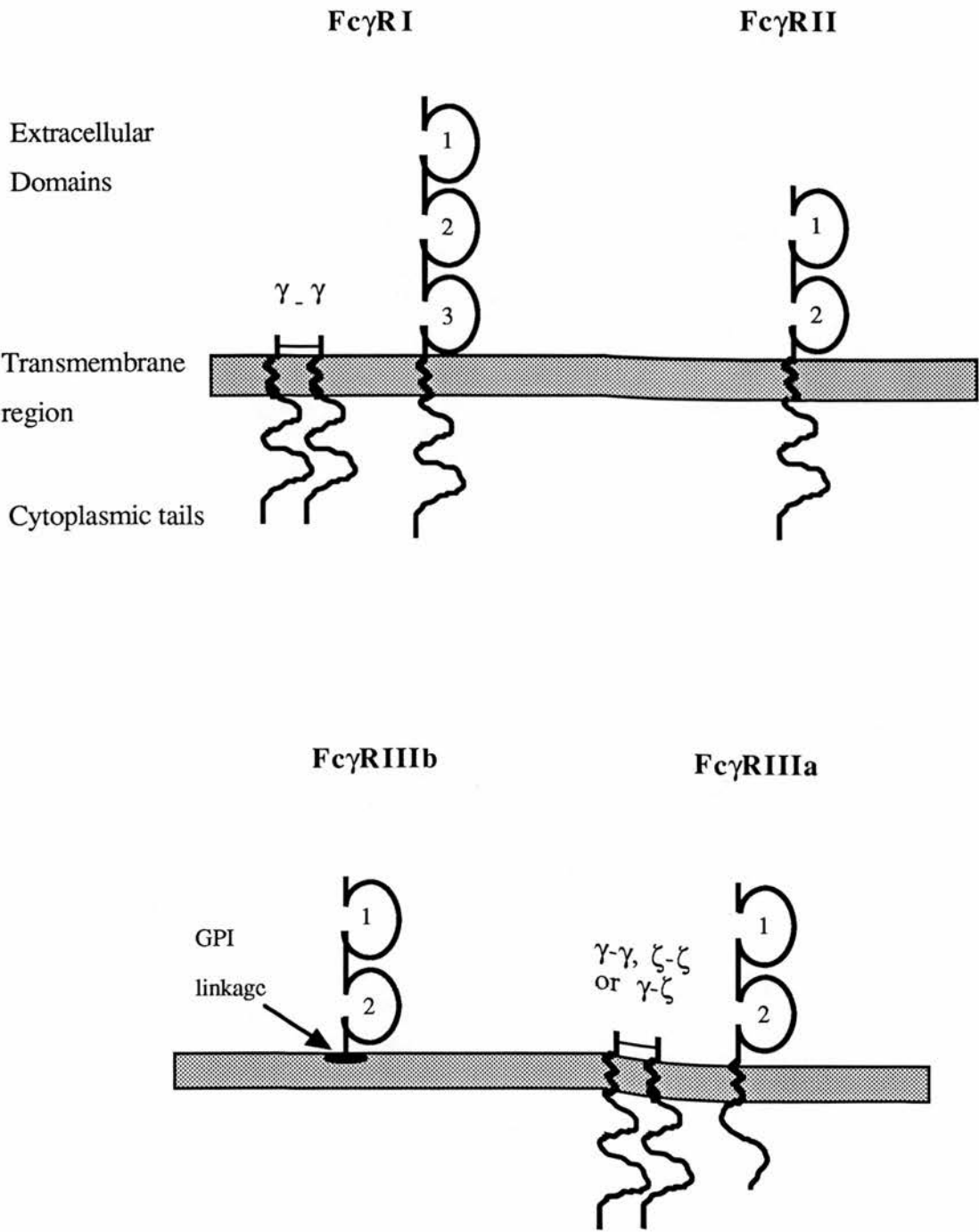
Soluble forms of Fc γ R have been demonstrated for all three classes in human sera. These molecules lack transmembrane regions and may be generated by protease activity and release from the cell surface (Huizinga et al 1990), or alternatively Fc γ R may be synthesised without the necessary transmembrane region and subsequently exported from the cell (Tartour et al 1993).

The area of Fc γ RII involved in interaction with the Fc portion of IgG was determined in experiments with chimeric Fc receptors. Binding was shown to be dependent on the second extracellular domain (ED 2); replacement of ED 2 of Fc ϵ RI with the ED 2 of Fc γ RII resulted in production of a chimera that bound IgG (Hulett et al 1991). The binding area was identified more specifically as residues 146 to 169 of Fc γ RII.

Figure 1

The schematic structure of the human Fc γ Rs is illustrated. Fc γ RI may have an associated γ - γ subunit, as shown; however it can also be expressed in the absence of this dimer. Fc γ RIIIa may have a γ - γ , a ζ - ζ or a γ - ζ dimer situated with it, while the GPI-linked form, Fc γ RIIIb, has no associated subunits.

FIGURE 1 Schematic Structure of Human Fc γ Receptors



1.10.2. Cellular Distribution

1.10.2.1. Fc γ RI

Fc γ RI is constitutively expressed by monocytes and macrophages and is inducible on neutrophils and eosinophils (Van de Winkel & Capel 1993). Three homologous genes have been identified in humans, encoding a receptor of molecular weight 72kD (Van de Winkel & Anderson 1991). A single gene has been described in the mouse (Sears et al 1990), and a bovine gene for Fc γ RI has recently been cloned and sequenced (Symons & Clarkson 1992). Amino acid sequences are conserved between these species, with 58% identity between the human, murine and bovine extracellular domains. In human monocytes and U937 cells, a γ - γ subunit has been identified associated with Fc γ RI, although this homodimer is not essential for expression of the receptor (Van de Winkel & Capel 1993, Masuda & Roos 1993).

1.10.2.2. Fc γ RII

Fc γ RII is the most widely distributed of the Fc receptors for IgG and is often the only type expressed by a particular cell. It is found on monocytes, macrophages, neutrophils, basophils, eosinophils, B cells, platelets and LC as well as DC from human blood (Thomas et al 1993a) and at low levels on DC from murine spleen (Crowley et al 1990). The receptor has a molecular weight of 40kD and in humans three genes have been identified encoding six isoforms (Qiu et al 1990). These are almost identical in their extracellular sequence but differ in transmembrane and cytoplasmic regions. Different genes may give rise to Fc γ RII of similar structure but differing functions (see below). Fc γ RII was not thought to be associated with the γ - γ subunits located with Fc γ RIII and sometimes Fc γ RI; however a recent study reports the association of 20% of cell surface Fc γ RII with γ - γ homodimers in cultured human monocytes (Masuda & Roos 1993).

1.10.2.3. FcγRIII

In humans, FcγRIII is encoded by two genes giving rise to distinct products. The gene FcγRIIIA encodes a receptor with a conventional transmembrane section and cytoplasmic region. This form is located on macrophages, NK cells and a subpopulation of T cells and monocytes. The FcγRIIIB gene product is expressed by neutrophils and eosinophils and has a glycosyl phosphatidyl-inositol (GPI) linkage to the cell membrane (Van de Winkel & Anderson 1991). FcγRIII is 50-80kD in size with the range due to extensive glycosylation. FcγRIIIA is associated with various disulphide-linked subunits associated as homo- or hetero-dimers ($\gamma\gamma$, $\gamma\zeta$ or $\zeta\zeta$), which are necessary for expression of the receptor (Fig.1). In contrast, FcγRIIIB can be found without these subunits (Kurosaki & Ravetch 1989). FcγRI is also associated with $\gamma\gamma$ -homodimers but can be expressed in the absence of the subunit (Van de Winkel & Capel 1993). These subunits are thought to have a role in signalling on occupancy of the Fc receptor, and different combinations of γ and ζ chains could represent alternative signalling pathways. $\gamma\zeta$ subunits are related to those expressed with FcεRI and the T and B cell antigen receptors, suggesting that all these associated chains may have a role in the internalisation of multimeric receptors.

FcR for different Ig classes were thought to be distinct; however one study reports that murine FcγRII and FcγRIII can bind IgE with a similar affinity to IgG (Takizawa et al 1992). Binding of IgE was functional and resulted in mast cell activation and serotonin release. In some parasitic diseases IgE-containing immune complexes are present *in situ*; binding of these complexes to low affinity receptors for IgG could activate cells such as macrophages, monocytes and lymphocytes.

1.10.2.4. FcγR Expression by T Lymphocytes

Reports of FcγR distribution on T cells have been contradictory; in general, these cells are thought to express FcγR only after activation (Sandor & Lynch 1993). Thus

Fc γ RII and Fc γ RIII have been detected on murine T cells (Daeron et al 1988). Mantzioris and colleagues (1993) have recently demonstrated Fc γ RII on a subset of T cells isolated from human peripheral blood. They found that 10-14% of CD4+ T cells and 27-69% of CD8+ T cells stained with monoclonals to CD32, and mRNA for this receptor was also demonstrated in CD8^{high} cells by polymerase chain reaction (PCR) analysis. Regulation of B cell IgG production by Fc γ R-expressing T hybridoma cells has been reported; the Fc γ RII on peripheral T cells could have a similar role. Soluble Fc γ RII can act as a binding factor for IgG, and has also been detected in the supernatants of T cell cultures (Sautes et al 1991).

1.10.3. Functions

Fc receptors are involved in many regulatory and effector mechanisms in the immune response including phagocytosis, antibody-dependent cell-mediated cytotoxicity (ADCC), the release of inflammatory mediators, regulation of Ig production and the enhancement of antigen presentation (Anderson et al 1990, Fanger et al 1989, Anegon et al 1988, Debets et al 1990, Klaus et al 1987). The latter is discussed in more detail below.

Cross-linking of Fc receptors is required for the above functions; this can lead to stimulatory or inhibitory signals depending on the cell type and perhaps the type or isoform of Fc γ R. For example, cross-linking of Fc γ RII on macrophages delivers a positive signal to the cell and the receptor and ligand are internalised for degradation and subsequent presentation in immunogenic form (Macintyre et al 1988). In contrast, occupation of the B cell Fc γ RII with ligand provides an inhibitory signal and the receptor is not internalised. Indeed, cross-linkage of Fc γ R with membrane immunoglobulin (mIg) on the B cell surface inhibits Ig production (Phillips & Parker 1984), and has been proposed as a regulatory mechanism for B cell responses.

A genetically determined polymorphism of human Fc γ RII has been demonstrated on monocytes, macrophages and neutrophils. This was initially discovered by differences

in the ability of cells from certain individuals to bind murine IgG1. Monocytes from approximately 70% of Caucasians bound murine IgG1 and supported T cell mitogenesis induced by murine IgG1 anti-CD3 monoclonal antibodies (high responders, HR), while those from the remaining 30% did not (low responders, LR). Only the LR receptor allotype is capable of binding human IgG2, while human IgG1 and IgG3 can be bound by both HR and LR (Van de Winkel & Capel 1993). The basis of the polymorphism is a single amino acid change in the second extracellular domain (Warmerdam et al 1990). The significance of this polymorphism is unclear, although an effect on the immune response to bacterial polysaccharide antigens has been suggested, as the antibodies produced against these antigens are mainly of the IgG2 isotype (Van de Winkel & Anderson 1991).

The affinity of Fc γ RII on human monocytes for complexed IgG is markedly increased by proteolysis, while the number of receptors on the cell surface remains constant. IgG-mediated functions of monocytes, such as the release of TNF- α , are also increased by proteolysis (Debets et al 1990). It is therefore possible that proteases released by cells such as granulocytes, activated T cells and macrophages *in vivo* could increase the affinity of Fc γ RII especially at areas of inflammation, with a corresponding increase in FcR-mediated functions (Tax & Van de Winkel 1990).

Fc γ RIII type A, found on NK cells and macrophages, is capable of mediating ADCC and phagocytosis, while Fc γ RIIIB on neutrophils, with its GPI linkage, is not. However, the latter may act synergistically with other FcR to mediate these functions, or alternatively it could be a means of trapping immune complexes without subsequent cell activation (Selvaraj et al 1988).

1.10.4. Signalling via FcR

Aggregation of Fc γ R is required before Fc γ R-mediated functions other than uptake occur. An increase in the intracellular concentration of calcium ions occurs following Fc γ R cross-linking (Van de Winkel et al 1990), although the events involved in

signalling on Fc γ R occupation have not been determined. Recent studies have investigated signalling and internalisation of Fc γ RIII on different cell types. The γ and ζ subunits situated with this Fc γ R do not possess intrinsic enzymatic activity but are associated with a 70kD tyrosine phosphoprotein, and receptor triggering induces increased association of this phosphoprotein with the ζ subunit in both T and NK cells (Vivier et al 1993). A similar protein located with the ζ subunit in T cells has been identified as a tyrosine kinase, and the authors suggest that the FcR-associated dimers interact with this non-receptor tyrosine kinase in initiation of signalling and cell activation. Greenberg and colleagues demonstrated the requirement for tyrosine phosphorylation in FcR-mediated phagocytosis in mouse macrophages (Greenberg et al 1993). Localised areas of tyrosine phosphorylation were seen at points of attachment of IgG-coated erythrocytes; increased concentrations of F-actin were seen in the same locations. Fc γ RIII was thought to be responsible for phagocytosis, with the γ or ζ subunits essential for signal transduction in FcR-mediated phagocytosis. Tyrosine phosphorylation was also required for the internalisation of Fc γ RII and Fc γ RI in a human monocytic cell line (Ghazizadeh & Fleit 1994). Fc γ RII was not tyrosyl phosphorylated on Fc γ RI stimulation, suggesting that these two receptors employ distinct signalling pathways.

The consequences of signalling via Fc γ R on neutrophils and macrophages include the release of inflammatory mediators such as leukotrienes and prostaglandins, and the inflammatory cytokines IL-1, IL-6 and TNF- α (Debets et al 1990, Krutmann et al 1990, Chantry et al 1989). The expression of cell surface molecules involved in interactions with other cells and the extracellular matrix may also be regulated after signalling through Fc γ R.

1.10.5. Regulation of Fc γ R Expression

Several cytokines have effects on Fc γ R expression. IFN- γ upregulates Fc γ RI on macrophages, although functions are differentially regulated with a decrease in phagocytosis contrasting with enhanced effector functions such as the oxidative burst

(Jungi et al 1989). IFN- γ also induces expression of Fc γ RI on neutrophils, which do not constitutively express this receptor (Fanger et al 1989).

Fc γ RII expression on macrophages is not increased by IFN- γ , although functions are affected as for Fc γ RI (Jungi et al 1991). The varying functional effects suggest a divergence of the signal transduction pathway after ligand binding to the FcR, with effector functions such as oxidative burst and ADCC promoted by IFN- γ while phagocytosis is inhibited. TNF- α and GM-CSF also enhance effector capabilities by increasing the killing capacity of cultured monocytes through Fc γ RII (Erbe et al 1990).

1.10.6. Identification of Fc γ Rs

There are currently several mAbs to each of the main Fc receptor types in humans and mice, although none are available for sheep Fc γ Rs. Anderson used IgG coupled to Sepharose to affinity purify human Fc γ Rs from lysates of alveolar macrophages and U937 cells. Two molecules were eluted from the immunoabsorbent, of molecular weight 40-43 and 72kD. These represented Fc γ RII and Fc γ RI respectively, and in further experiments polyclonal antisera (Anderson et al 1985) and monoclonal antibodies against Fc γ RI and Fc γ RII were raised (Anderson et al 1986).

1.11. Receptor-mediated antigen uptake

An accessory cell must be capable of taking up exogenous antigen, processing the antigen to a form immunogenic for T lymphocytes, presenting the peptides generated in a form recognisable by the TCR and providing the costimulatory signals necessary for activation of the responding cell. The amount of antigen displayed on the accessory cell surface is dependent largely on the ability of the cell to accumulate antigen and the quantity of MHC class II products available for interaction with peptides produced by processing (Lanzavecchia 1990). Non-specific methods of antigen uptake include adsorption and fluid phase endocytosis; the efficiency of antigen capture is greatly increased if a cell surface receptor is employed.

1.11.1. Enhancement of antigen presentation

The targeting of antigens to cell surface molecules can facilitate subsequent antigen presentation. For example, Snider and Segal (1987) found that enhanced presentation followed when antigen was targeted to class I and class II MHC molecules on B cells and macrophages, and membrane IgD on B cells. Other surface molecules such as the B cell differentiation antigen B220 were ineffective. This suggests that several surface molecules can act as receptors for antigen uptake.

MIg on the B cell surface interacts with specific antigen which is then presented with great efficiency to responder T cells. Internalisation of mIg/ antigen and processing are necessary before T cells can be activated in secondary responses (Lanzavecchia 1985); mIg therefore acts only as a receptor. In contrast, B cells are poor presenting cells for antigens taken up non-specifically, probably due to their low capacity for pinocytosis (Chestnut et al 1982).

Antigen-specific antibody can target attached antigen to Fc receptors on the surface of macrophages, B cells and DC. Celis and Chang (1984 a&b) found that T cell clones specific for hepatitis B surface antigen (HBsAg) could be activated by 10-100 times less antigen in the presence of specific antibodies and FcR-expressing APC. An intact Fc portion of antibody was required for this enhancement. Similar evidence has been reported by Schalke et al (1985) using the acetylcholine receptor as an antigen; in this case the APC responsible was the splenic DC. Amigorena and colleagues transfected Fc γ RIII into FcR-negative B lymphoma cells. These APC showed enhanced presentation of antigen to specific T cells; 30-100 fold less antigen was required for T cell activation when antigen was complexed with IgG (Amigorena et al 1992).

Studies by Marusic-Galesic et al (1991, 1992) investigated T cell responses to antigen in immune complex form both *in vitro* and *in vivo*. *In vitro*, primed, polyclonal murine T cells were cultured with human serum albumin (HSA) alone or complexed with specific antibody. Up to 100-fold less HSA was needed for T cell activation in complex form and mAbs to Fc γ R blocked the enhancement. In the *in vivo* work, the T cell

response elicited by HSA in immune complexes was equivalent to that seen with five times as much soluble antigen.

The antigen/ antibody ratio was shown to be important in that complexes at equivalence or moderate antibody excess provided optimal T cell stimulation (Manca et al 1991). In this case, extreme antibody excess did not potentiate T cell activation at suboptimal antigen levels, and actually inhibited proliferation at stimulatory antigen concentrations. Immune complexes were still taken up in antibody excess, and it was suggested that an excess of polyclonal antibodies could prevent proteolytic degradation *in vivo*, acting as a mechanism to regulate the T cell response.

1.11.2. Internalisation of FcR-Ligand

Some surface receptors such as that for transferrin are internalised constitutively (Watts 1985), while others, including Fc γ R, first require a ligand binding signal (Ukkonen et al 1986). The fate of immune complexes bound to Fc γ RII varies with cell type. In mice, different isoforms of Fc γ RII are expressed by macrophages and B lymphocytes. The macrophage receptor is internalised via clathrin coated pits when ligand binding occurs, while the B cell receptor is not (see below).

The pathway followed by the receptor-ligand complex once internalised depends on the ligand; while a monovalent Fab fragment of an anti-Fc γ R mAb is recycled to the cell surface still bound to the receptor, multivalent ligands such as immune complexes do not recycle and enter the late endosomal or lysosomal compartments for degradation (Ukkonen et al 1986). Because the Fc γ R is also degraded, binding of ligand to Fc γ R will result in a rapid downregulation of the receptor. Association of antigenic peptides with MHC class II molecules may follow in the late endosomal or lysosomal compartments (see above).

In contrast to the macrophage isoform of Fc γ RII, that found on murine B cells, which differs in the cytoplasmic region, is not internalised upon ligand binding (Miettinen et al

1989). While binding of ligand to Fc γ RII on macrophages provides a positive signal to the cell, cross-linking of mIg and Fc γ RII on the B cell surface inhibits cell activation. This may act as a regulatory mechanism *in vivo* to control the production of specific antibodies.

Studies of Fc γ RIII have identified the area required for internalisation of this receptor. This was found to be a cytoplasmic region of the γ - γ subunit containing a signal sequence incorporating two tyrosine residues (Amigorena et al 1992).

1.12. Sheep Afferent Lymph DC

Cannulation of both efferent and afferent lymphatics of sheep has been performed routinely for many years (Hall & Morris 1962, Miller & Adams 1977). DC are found only in afferent lymph, but there are technical difficulties in obtaining these cells in any quantity from the multiple afferent lymphatics which are much smaller in diameter than the efferent lymphatics. Pseudoafferent cannulations give an improved lymph flow with decreased risk of clotting, and are carried out after resection of the lymph node and a time period to allow anastomosis of the former afferent and efferent ducts (Hopkins et al 1989). The former efferent duct is then cannulated. The cannula is heparinised before use to further reduce the possibility of clot formation, and lymph is collected into sterile bottles containing heparin and antibiotics, as described in Chapter 2.

This procedure allows the direct investigation of immune responses *in vivo* by analysis of phenotype and function of freshly isolated cells after the administration of antigen into the drainage area of the cannulated duct. 1-10% of cells in afferent lymph are DC, and these can be separated to a reasonable level of purity by one centrifugation over metrizamide. A higher degree of purity can be achieved if a second metrizamide separation is employed. Isolation is therefore simple, and does not require repeated culture steps which could result in modification of DC morphology and function. With an average of 5×10^4 DC per ml of afferent lymph, sufficient numbers of these cells for functional assays can be obtained from an overnight collection. Lymph from one animal can be obtained for several weeks.

1.13. Aims of This Thesis

Previous work with afferent lymph DC of sheep has demonstrated their immunostimulatory ability in the presentation of both soluble and alloantigens. After antigen administration in a primed sheep, MHC class II is upregulated on the DC surface, and this corresponds with increased ability of DC to present a second antigen. CD1 expression is also increased (Hopkins et al 1989). DC take up fluorescent antigen more efficiently in the presence of intact specific antibody, both *in vitro* and *in vivo*, and form rosettes with IgG-coated erythrocytes (Harkiss et al. 1990). Fc receptors for IgG have thus been proposed as a mechanism of immune complex uptake by sheep afferent lymph DC in secondary immune responses.

The aims of this thesis were:-

- 1) The characterisation of DC surface markers by flow cytometry analysis in primary and secondary immune responses *in vivo* and *in vitro*, including molecules with functions in antigen presentation, adhesion and costimulation. Any modulation of these surface markers could be important in the migration of DC and subsequent activation of antigen-specific T cells.
- 2) Investigation of the expression of Fc γ R by afferent lymph DC, from resting lymph and after antigen challenge. This necessitated the generation of anti-ovine Fc γ R antibodies for analysis of DC populations by flow cytometry and Western blot techniques.
- 3) To determine the role of Fc γ R on DC in antigen uptake and presentation, by measuring T cell proliferation in response to soluble or complexed antigen presented by DC. From previous work, Fc γ R-bearing APCs would be expected to present antigen in the form of immune complexes with increased efficiency; this was initially tested with unfractionated ovine PBMs containing Fc γ R-expressing cells such as monocytes, B cells and DC as well as responding T cells.

CHAPTER TWO

MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1. Antigens

Ovalbumin (OVA) and human serum albumin (HSA) were obtained from the Sigma Chemical Company, Poole, Dorset.

2.2. Animals and Surgery

2.2.1. *Sheep*

Finnish Landrace or Scottish Blackface sheep were obtained from the Moredun Research Institute, Edinburgh. Animals were immunised with 1mg OVA in complete Freund's Adjuvant (CFA) administered subcutaneously and followed at four-weekly intervals by 2 further injections of 200µg OVA in incomplete Freund's Adjuvant (IFA). A similar procedure was used for priming sheep to HSA. Sera were tested for anti-OVA or anti-HSA antibody activity by enzyme-linked immunosorbent assay (ELISA) as described in section 2.7.2.

To obtain IgM anti-OVA antibodies, OVA was administered to sheep on rabbit erythrocytes (Torrighiani & Roitt 1965). Rabbit erythrocytes were washed in PBS and resuspended as a 50% solution in tannic acid, before mixing at room temperature for 20 minutes. After washing again, the erythrocytes were resuspended as a 10% solution in PBS and 50mg OVA was added per 100ml of solution. The mixture was incubated at 37°C for 30 minutes, centrifuged at 400g for 5 minutes and resuspended in 20ml PBS. 6ml of cells in PBS was administered to sheep by the intravenous route, daily for three consecutive days. Animals were bled to obtain serum on days 9, 10 and 11 after the initial antigen administration.

Surgical procedures were carried out under general anaesthesia, induced with intravenous alphaxolone/ alphadolone ("Saffan", Pitman-Moore, Crewe). Sheep were then intubated and anaesthesia was maintained with halothane (Halothane-M&B, Rhône

Mérieux Ltd, Harlow) and nitrous oxide. Pseudoafferent cannulations of the prefemoral ducts were carried out by Dr J. Hopkins (Hopkins et al 1985). Prefemoral lymph nodes were resected and a period of at least six weeks was allowed for the anastomosis of afferent lymphatics with the former efferent duct, which was then cannulated. Initially untreated cannula was used (internal diameter 0.58mm, Portex, UK), while in later experiments the same cannula was heparinised to reduce clotting and improve lymph flow. Heparinisation was carried out as follows:- the cannula was flushed with acetone, followed by 2% Vectabond (Vector Laboratories, Peterborough) in acetone. The latter was left in the cannula for five minutes at room temperature before a repeat flush with acetone. The cannula was then filled with neat heparin (5000iu per ml, Leo Laboratories, Princes Risborough) and both ends were clamped. Before insertion into a prefemoral duct, the clamps were released and the cannula was wiped with absolute alcohol.

Cannulated animals were housed in metabolism cages, and lymph was collected into sterile bottles containing 2.5×10^3 units of heparin and 2.5×10^4 units of penicillin and streptomycin. Overnight collections were generally used for experiments with DC.

2.2.2. *Rabbits*

Rabbits were bred in the Department of Veterinary Pathology Animal House. Animals were immunised with 250 μ g of synthetic peptide antigens conjugated to OVA at a 1:1 ratio (section 2.10), and emulsified in Hunter's Titermax (Sigma). Antigen was administered in several sites subcutaneously. Further injections of the same amount of peptide/ OVA conjugate were given 10 and 17 days after the initial immunisation. Rabbits were then bled from the ear vein to obtain sera for testing.

2.3. Separation of Cell Populations

2.3.1. *DC from afferent lymph*

DC were obtained from afferent lymph by centrifugation over metrizamide (Nycomed,

Oslo). Afferent lymph cells were spun down and resuspended in RPMI medium containing 10% FCS (Flow Laboratories, Herts). Cells were then layered over 14.5% metrizamide in the same medium and centrifuged at 600g for 15 minutes at 4°C. The cells collected from the interface were 60-80% DC by morphology when examined on Leishmans-stained cytopins (Fig.1). Cells were washed twice in suitable medium before further use.

A DC population of increased purity was obtained for Western blot analyses, by adding a second metrizamide separation. The interface cells were 90-95% DC by morphology as above. These cells were used to make DC lysates for blots (section 2.7.6.).

2.3.2. Peripheral Blood Mononuclear Cells (PBMs)

Blood was defibrinated by shaking with glass beads, or collected into tubes containing 10 units of heparin per ml of blood, then diluted with phosphate-buffered saline (PBS). Lymphoprep (Nycomed) was layered under the diluted blood and tubes were subjected to centrifugation of 950g for 20 minutes at room temperature. PBMs were harvested from the top of the Lymphoprep layer and washed twice by centrifugation at 500g in medium before counting.

2.3.3. White Blood Cells (WBCs)

WBCs were obtained from whole blood by the lysis of erythrocytes. Three volumes of lysis solution (0.14M NH₄Cl, 0.017M Tris, pH 7.2) were added to one volume of defibrinated or heparinised blood at 37°C. After a 4 minute incubation, cells were spun at 400g, washed by further centrifugation in medium and counted.

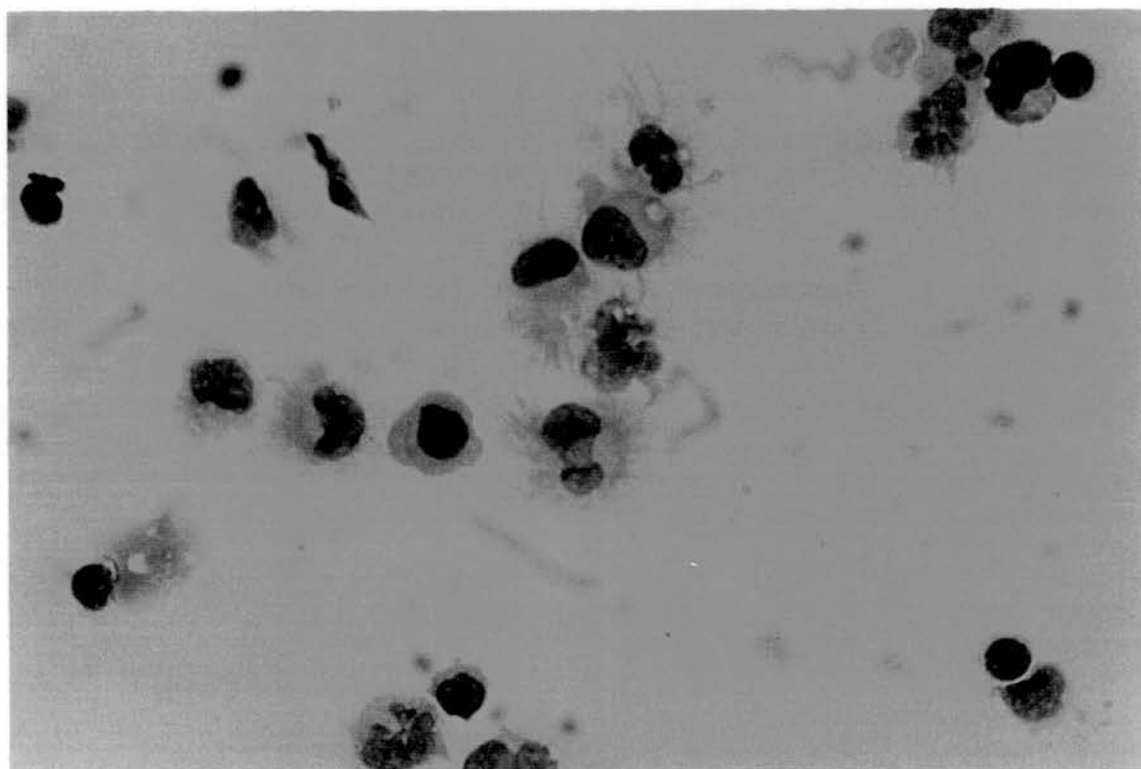
2.3.4. CD4+ T cells

PBMs were washed twice in PBS then resuspended in PBS at a concentration of 4×10^7 cells per ml. Biotinylated anti-CD4 monoclonal antibody was added to a predetermined

Figure 1

Afferent lymph cells were washed and resuspended in RPMI medium containing 10% FCS. The cells were layered over 14% metrizamide and subjected to centrifugation as described in section 2.3.1. The interface layer of cells was collected and examined on cytopins stained with Leishman's solution. DC were the larger cells with irregular shaped nuclei and abundant cytoplasm, with a veiled or dendritic appearance. Lymphocytes were identified as smaller cells with regular nuclei and only a small amount of visible cytoplasm.

FIGURE 1 Cytospin of DC Population Obtained After Separation Over Metrizamide



optimal concentration; cells were incubated on ice for 30 minutes. After one wash in PBS, the cells were resuspended in 90µl PBS per 10⁷ cells and streptavidin-labelled magnetic MACS microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) were added at 10µl per 10⁷ cells. Streptavidin-phycoerthrin (Sigma) was also added at this stage to allow assessment of the purity of the selected population by flow cytometry. A 15 minute incubation on ice followed before cells were washed in PBS, resuspended in 500µl 1% bovine serum albumin (BSA) in PBS and added to the equilibrated MACS column.

Columns were prepared by filling with ice-cold 1% BSA in PBS from the bottom and tapping gently to remove air bubbles. After placing on the permanent magnet, three column volumes of buffer were run through from the top of the column, using a 24 gauge needle to ensure the correct flow rate. Labelled cells were loaded onto the column and followed by three volumes of buffer. Negatively stained cells passed through the column matrix and were collected in a universal. The column was removed from the magnet, and buffer was flushed from the syringe up through the column matrix to dislodge the attached cells. After replacing the column on the magnet, buffer was allowed to flow out through the matrix. A 22 gauge needle was substituted for the 24 gauge one used initially, and three volumes of buffer were used to wash the column through from the top at an increased flow rate. The backflush and wash were repeated three times before removing the column from the magnet, and eluting positively stained cells by flushing with 20mls of 1% BSA in short pulses. Cells were spun down and counted. The purity of the selected population was determined on a Becton Dickinson FACScan (FACS). If <93% of cells were CD4+, they were passed through the column a second time to increase the quality of the separation.

MACS columns were regenerated by washing with 100mls sterile distilled water (SDW) followed by 100mls absolute alcohol, then dried under vacuum before storage. Where selected cells were to be cultured, columns were autoclaved and all procedures were carried out in a laminar flow hood.

2.3.5. *Alveolar macrophages*

These cells were obtained from bronchoalveolar lavage fluid (BALF) of sheep post mortem. Lungs were removed and lavaged with 1 litre HBSS containing 5mM ethylene-diamine tetraacetic acid (EDTA), followed by 1 litre PBS also containing EDTA. The resulting BALF was passed through a coarse wire mesh to remove mucus before spinning to pellet the cells. After being resuspended, the cells were separated over a 14.5% metrizamide gradient as described above for DC.

2.4. Monoclonal Antibodies and Flow Cytometry Analysis

2.4.1. *Monoclonal Antibodies*

The monoclonal antibodies used for surface phenotyping of cells in flow cytometry analysis are shown in Table 1.

2.4.2. *Cell Staining for Immunofluorescence Analysis*

Cells were suspended in PBS containing 2% BSA and 10mM sodium azide (PBA), and 10^6 cells in 50 μ l medium were added per LP2 tube. An equal volume of monoclonal antibody or dilution of antiserum was added, and the tubes were kept on ice for 30 minutes. After 3 washes by centrifugation in 500 μ l PBA, 50 μ l of relevant fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-labelled conjugate was added at a predetermined optimal concentration. A further 30 minute incubation on ice was followed by washes as above; the cells were then resuspended in 300 μ l PBS and analysed on a FACS. Where cells could not be analysed immediately they were fixed in 0.5% paraformaldehyde in PBS and stored protected from light at 4°C.

2.4.3. *Conjugates for Immunofluorescence analysis*

All of the conjugates used were obtained from Sigma:-

Sheep anti-mouse IgG FITC; goat anti-mouse IgG PE; goat anti-mouse IgM FITC;

Table 1

The various monoclonal antibodies used in the immunofluorescence analysis of ovine cells are listed in this table. SW73.2, RSh γ 1, RSh γ 2 and MS121 are rat monoclonals specific for ovine antigens, while all the other monoclonals are murine Igs. The monoclonal antibodies were used either as supernatants, or as dilutions of ascites. Where ascites preparations were used, they were titrated to obtain the optimum staining of ovine cells. A reference or the source of the monoclonal antibody is given in each case.

TABLE 1: Monoclonal antibodies used for cell surface phenotyping

Antibody	Specificity	Isotype	Reference/ Source
VPM 6	Ovine IgG	IgG1	Jones 1988
VPM 8	Ovine Ig light chain	IgG1	Jones 1988
VPM 13	Ovine IgM	IgG1	Moredun Research Institute
VPM 18	Ovine LCA (CD45)	IgG1	Hopkins et al 1990
VPM 19	Ovine MHC Class I	IgG1	Hopkins et al 1990
VPM 36	Ovine MHC Class II (DQ α)	IgG1	Dutia et al 1990
VPM 53	Campylobacter	IgG1	McOrist et al 1987
SW73.2	Ovine MHC Class II	IgG2a	Hopkins et al 1986
17D	Ovine CD4	IgG1	Mackay et al 1988a
SBU-T8	Ovine CD8	IgG2a	Maddox et al 1985
SBU-T6	Ovine CD1	IgG1	Mackay et al 1985
135A	Ovine CD2	IgG1	Giegerich et al 1989
L180/1	Ovine LFA-3	IgG1	Hunig et al 1985
F10/150/39	Ovine LFA-1	IgG1	Mackay et al 1990
HP2/1	Human VLA-4	IgG1	Sanchez-Madrid et al 1990
GoH3	Human VLA-6	IgG2a	Hemler et al 1988
DF5	Human CD29	IgG1	Ylanne et al 1989
25-32	Ovine CD44	IgG1	Mackay et al 1988b
RSh γ 1	Ovine IgG1	IgG2a	S. Hobbs, Institute of Cancer Research, Royal Marsden Hospital, Sutton, Surrey.
RSh γ 2	Ovine IgG2	IgG2b	
MS121	Ovine IgA	IgG2a	

goat anti-rat IgG FITC and goat anti-rabbit IgG FITC; streptavidin-PE.

The conjugates were diluted in PBA and titrated to obtain optimum dilutions before use.

2.4.4. Biotinylation of Monoclonal Antibodies

Approximately 2mls of ascites was applied to a Protein A-Sepharose column in order to purify IgG. Columns were run in PBS, and bound IgG was eluted with 0.1M glycine/HCl buffer pH 2.5, then neutralised with 2M Tris as described below. The eluted antibody was extensively dialysed into 0.1M sodium bicarbonate, pH 8.4, and its protein concentration was determined from the optical density (OD) at 280nm. 2mg biotin (Sigma) was dissolved in 1ml dimethyl sulphoxide (DMSO) immediately prior to use; 75µg biotin was then added per 1mg IgG and the mixture was rotated at room temperature for 4 hours. The antibody was dialysed into PBS/ azide and FACS analysis of titrations was carried out to determine the optimal concentrations for cell staining. The biotinylated antibody was stored in aliquots at -70°C until used.

2.4.5. Analysis of Flow Cytometry Data

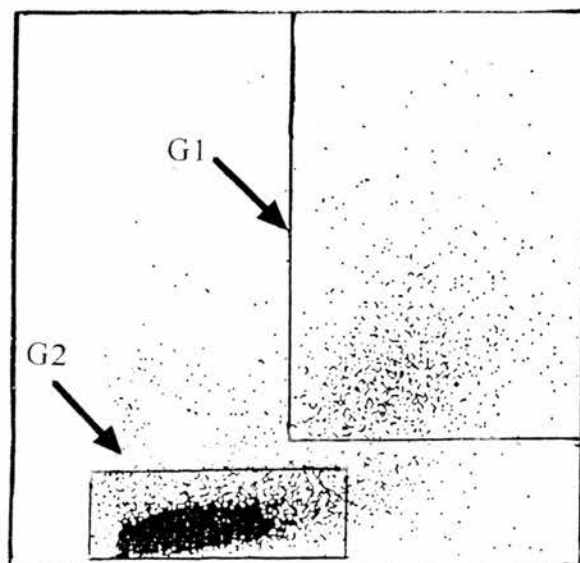
Cells were prepared for flow cytometry as described above. A representative FACS light scatter plot for afferent lymph cells is shown in Fig.2 (i). Side scatter, detected at 90° to the laser beam, is a result of reflection and refraction of the incident light, while forward scatter due to diffraction is detected along the axis of the beam and depends on the surface area of the cell. DC were gated on as cells with high forward and side scatter due to their large size and complexity. A minimum of five thousand DC were analysed per sample. The gate used for analysis of afferent lymphocytes is also shown in Fig.2 (i). WBCs obtained from defibrinated blood after the lysis of erythrocytes were used in some experiments; a scatter plot of these cells with the gates used for analysis of granulocytes and lymphocytes is shown in Fig.2 (ii). The gates used for the analysis of particular cell populations are difficult to set accurately; in each case there is the possibility of a minor percentage of contaminating cells of another population being

Figure 2

Afferent lymph cells were collected from a pseudoafferent cannulation of the prefemoral duct, while WBCs were obtained from whole blood by ammonium chloride lysis of erythrocytes. The cells were analysed by flow cytometry for their forward (FSC) and side (SSC) light scatter profiles. The live gates used to analyse selected cell populations are shown.

FIGURE 2 Scatter Profiles of Ovine Cells

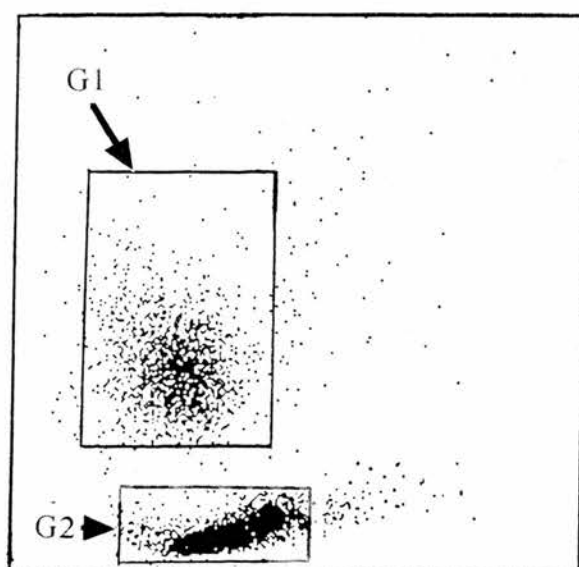
(i) Afferent Lymph Cells



G1 - DC

G2 - lymphocytes

(ii) WBCs



G1 - neutrophils

G2 - lymphocytes

included within the gate.

Negative controls were isotype-matched irrelevant monoclonals, second layer reagents only, or, for rabbit sera, dilutions of the preinoculation serum. The percentage of DC positive for each marker was assessed as the fluorescence staining over and above that of the negative control. DC are a heterogeneous population of cells, and negative control peaks often showed a "tail" of fluorescence as shown in Fig.3; this made it necessary to set markers with 5-10% of cells in the negative control sample positive. This percentage was then subtracted from the proportion of DC staining for each marker tested. Lymphocytes and neutrophils gave a sharper negative control peak and markers for these cells were set so that <1% of cells in the negative control peak showed positive fluorescence (Fig.3).

2.5. Antigen Uptake by DC

2.5.1. Production of FITC-OVA

OVA was dissolved in 0.25M sodium carbonate pH 9, 0.1M sodium chloride to give a protein concentration of 20mgml⁻¹. FITC in DMSO was added at 0.05mg per mg protein, for a one hour incubation at room temperature. The mixture was then applied to a Sephadex G-25 column equilibrated in PBS. Two coloured bands were visualised and the heavier of these, representing conjugated OVA, was collected. FITC-OVA was aliquoted and stored protected from light at -70°C.

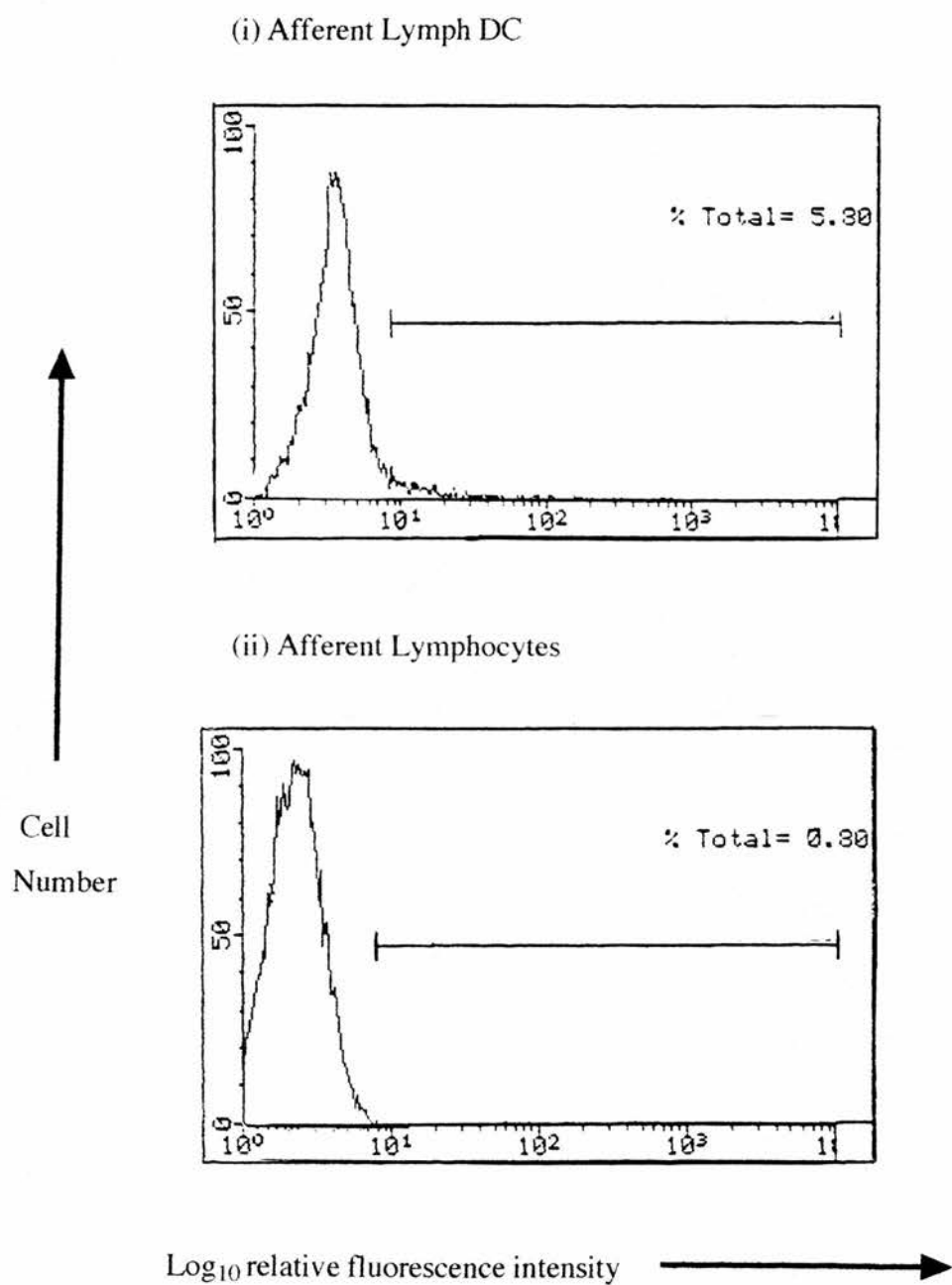
2.5.2. In Vitro uptake of antigen

Antigen uptake was investigated in primary and secondary responses *in vitro*. In experiments representing secondary responses, afferent cells were washed and resuspended in PBA. 100µl of afferent cells was then added to 50µl FITC-OVA dilutions in PBA. To some tubes 50µl of afferent lymph from an OVA-primed sheep was added as a source of anti-OVA antibodies. Lymph was first tested by ELISA to demonstrate the presence of anti-OVA antibodies of IgG isotype. In some experiments,

Figure 3

Afferent lymph cells were stained with a second layer reagent only (anti-mouse Ig-FITC) and analysed by flow cytometry on a FACScan. The DC and lymphocyte populations were gated on as described, according to their forward and side light scatter characteristics. Representative negative peaks are shown in (i) for DC and (ii) for lymphocytes. The markers were set as shown for analysis; generally 5-10% of DC were positive, and <1% of lymphocytes were positive, in the negative control populations.

FIGURE 3 Negative Control Flow Cytometry Profiles Of DC and Lymphocytes



affinity-purified antibodies (section 2.8.2.) were used instead of lymph fluid. Incubation at room temperature for 30 minutes was carried out in LP2 tubes. After washing three times in PBA, the cells were analysed by FACS for fluorescence. In some experiments unlabelled OVA was used and the cells were subsequently incubated with monoclonal antibodies to detect surface immunoglobulins. The uptake of FITC-OVA by alveolar macrophages in the presence or absence of specific antibodies of IgG isotype was also investigated using the above method.

To detect antigen uptake during primary responses, the above procedure was modified to include IgM anti-OVA antibodies instead of IgG antibodies. Serum was collected from sheep primed with OVA as described above, on days 9-11 of the primary immune response. Unfractionated serum or purified IgM antibodies were added to afferent cells with FITC-OVA as described above.

2.5.3. Antigen Uptake In Vivo

1mg FITC-OVA in PBS was injected intradermally into the drainage area of a cannulated prefemoral pseudoafferent duct in a sheep repeatedly primed with OVA. Lymph was collected at various time points and the cells were stained with monoclonal antibodies followed by a phycoerythrin conjugate before analysis by FACS. In some experiments unlabelled OVA was injected and the afferent cells were stained with monoclonal antibodies followed by a FITC conjugate.

2.6. DC Phenotype Analysis After Antigen Stimulation

2.6.1. In Vitro Experiments

Unseparated afferent cells from a primed sheep were cultured with medium only, OVA at $1\mu\text{gml}^{-1}$, or OVA at $1\mu\text{gml}^{-1}$ and specific affinity purified antibodies at $30\mu\text{gml}^{-1}$. Antigen and antibody were added to 24 well plates and incubated at 37°C for thirty minutes to allow immune complex formation. Afferent cells were washed twice in

medium before addition to wells at a concentration of 10^6 cells per ml. The cells were stained for FACS analysis before culture and after 2 and 8 hour incubation periods; DC were gated on as described above. The viability of the cells at each time point was determined as >99% by Trypan Blue exclusion.

2.6.2. *In Vivo* Experiments

1mg of OVA in 1ml PBS was injected intradermally in two or three sites, into the drainage area of a cannulated OVA-primed sheep. Afferent lymph was collected at various time points after antigen administration, and the cells were washed and stained for FACS analysis as described above. DC were gated on as before.

2.7 Protein Immunochemistry

2.7.1. *Preparation of F(ab')₂ Antibody Fragments*

Affinity purified IgG anti-OVA antibodies were dialysed into 0.1M sodium acetate pH 4.5. Pepsin (Sigma) was made up in the same buffer and added at 3% (w/w). Digestion took place overnight at 37°C, with constant rotation of the mixture; the digestion was stopped by neutralisation with 2M Tris. F(ab')₂ fragments were separated from residual whole IgG by DEAE-cellulose ion-exchange chromatography (DE52, Whatman, Maidstone). Antibodies were dialysed extensively into 10mM sodium phosphate pH 7.5 and applied to columns of equivalent pH and conductivity. Whole IgG molecules were retained on the column while F(ab')₂ fragments passed through and were collected. The various fractions were analysed by sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) to ensure that no intact IgG molecules remained (see Chapter 5). Antibody activity was confirmed by ELISA as described below, and preparations were dialysed into RPMI before use in assays.

2.7.2. Assessment of antibody activity and isotype by ELISA

Microtiter plates (Immulon 1, Dynatech Laboratories Ltd, Virginia) were coated overnight with antigen in PBS at a concentration of 10µgml⁻¹. The plates were washed in PBS / 0.5% Tween 20 before the addition of antibody dilutions in PBA. PBA alone served as a negative control. After a one hour incubation at room temperature, the plates were washed again then a second antibody or a relevant conjugate was added. For the detection of sheep IgG, an anti-sheep IgG alkaline phosphatase conjugate at a dilution of 1/1000 was the next layer, with a similar method used for rabbit IgG. To measure the anti-OVA antibody activity of F(ab')₂ preparations VPM 8 was employed to detect sheep immunoglobulin (Ig) light chains, followed by an anti-mouse IgG alkaline phosphatase conjugate. All of the conjugates were obtained from Sigma.

The antibody isotype in ovine preparations was investigated using the following second layer monoclonals:-

- RShγ1 against ovine IgG1
- RShγ2 against ovine IgG2
- VPM 13 against ovine IgM

These were followed by an appropriate alkaline phosphatase conjugate, with incubation for 30 minutes at room temperature. The plates were washed once more, then alkaline phosphatase substrate (Sigma 104 phosphatase substrate) , made in 0.1M glycine, 0.05M NaOH, 0.5mM MgCl₂, 0.5mM ZnCl₂ was added as the final layer. The plates were read after a 30 minute incubation at 37°C protected from light, using a 405nm filter. Positive results were those greater than the mean + 2 standard deviations of the negative control wells.

2.7.3. Gel Electrophoresis

SDS-PAGE was used for protein analysis. 5% and 20% acrylamide solutions were

made in 0.38M Tris, 2mM EDTA pH8.8 with 0.13% SDS. To 4.2ml of each solution was added 3µl *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Stratagene, La Jolla, California) and 30µl 10% ammonium persulphate, before pouring to form a gradient slab gel.

The stacking gel was 2.5% acrylamide, 0.13M Tris pH 8, 0.1% SDS. 60µl ammonium persulphate and 20µl TEMED were added to 10mls of solution before pouring the stack. The protein samples were boiled for three minutes in an equivalent volume of SDS reducing buffer (25mM Tris, 2% SDS, 20% glycerol, 0.01% bromophenol blue and 5% mercaptoethanol), before loading onto gels. Low molecular weight markers of 94, 67, 43, 30, 20.1 and 14.4 kD (Pharmacia, Uppsala, Sweden) were used. Gels were run at 200V for 45 minutes in a tank buffer of 50mM Tris, 384mM glycine and 0.1% SDS, then stained to visualise proteins.

2.7.4. SDS-PAGE Staining

2.7.4.1. Silver stain

The SDS-PAGE gels were fixed in 50% methanol, 10% acetic acid for 30 minutes, then in 5% methanol, 7% acetic acid for the same period and finally in 10% glutaraldehyde solution. After washing overnight with deionised water, 0.1% silver nitrate was added for 15-30 minutes. The stain was rinsed off with deionised water followed by developer (3% sodium carbonate with 50µl 37% formaldehyde per 100ml), then more developer was added. Once the protein bands were clearly visible the reaction was stopped by the addition of solid citric acid. The gels were washed for at least 30 minutes in deionised water, then fixed in 10% Ilfofix for 1 minute and dried down immediately.

2.7.4.2. Coomassie blue stain

This stain was used where the amount of protein present was estimated to be at least

5µg per band. A solution of 0.25% coomassie blue, 20% methanol and 5% acetic acid was prepared and filtered before use. The gels were stained for 30 minutes, rinsed in deionised water and de-stained in 20% methanol, 5% acetic acid until protein bands could be visualised. Drying of the gels was then carried out as above.

2.7.5. Western Blot Analysis

A semi-dry electric blotter (Ancos, Denmark) was used for this procedure. Six pieces of 3mm chromatography paper (Whatman) were soaked in blotting buffer (25mM Tris, 40mM glycine, 20% methanol) and placed on the bottom carbon plate. A nitrocellulose layer (Hybond C, Amersham) was placed on top, then the gel and finally another three pieces of wet paper. Blotting was carried out at 150mA for two hours.

Prestained low molecular weight markers (BRL, Uxbridge) were removed and the rest of the blot was blocked in PBS containing 3% BSA, 0.5% Tween 80 for at least 1 hour. Serum/ antibody dilutions in blocking buffer were added for an overnight incubation. Several washes in PBS/ 0.5% BSA preceded the addition of the anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1/1000 in blocking buffer. After a 1 hour incubation and further washes in PBS/ 0.5% BSA, a final wash of 0.1M Tris pH 9.5 was carried out before the addition of developer. Blots were developed with nitroblue tetrazolium and bromo-chloro-indolyl phosphate (BCIP) in 0.1M Tris pH 9.5, with added magnesium chloride (Pluzek & Ramlau 1988). The reaction was stopped by washing the blots with water.

2.7.6. Preparation of Alveolar Macrophage Lysate

Alveolar macrophages were obtained from the lungs of sheep at post mortem as described above. The cells were washed twice in PBS and resuspended at $5 \times 10^7 \text{ml}^{-1}$ in lysis buffer of 1% NP-40 in PBS with the following protease inhibitors: 0.5mM benzamidine, $5 \mu\text{gml}^{-1}$ leupeptin, $2 \mu\text{gml}^{-1}$ pepstatin and 1mM polymethylsulphonyl fluoride (PMSF). Cells were lysed on ice for 30 minutes before centrifugation at 12,000g for 30 minutes to remove cell debris. Sodium azide (10mM) was added to

supernatants which were stored at -70°C. The preparation of lysates of other cell types (DC, lymphocytes) for use in Western blots was carried out using the same method.

2.7.7. Ouchterlony Double Gel Diffusion

This method was employed to ascertain equivalence points for antigens and specific antibodies prepared from the sera of primed sheep. Gels of 1% agarose in PBS were poured and wells of 4mm diameter cut in a radial pattern. Antigen was added to the central well and dilutions of antibody to the outer wells. After overnight incubation in a humidified atmosphere at 4°C, equivalence was determined as the sharpest line of precipitation between the central and an outer well.

2.8. Affinity Chromatography

2.8.1. Coupling proteins to Cyanogen bromide-activated Sepharose

This method was used to attach IgG, HSA, BSA and OVA to the Sepharose matrix. The protein to be coupled was dissolved or extensively dialysed into coupling buffer (0.1M NaHCO₃, 0.5M NaCl, pH 8.3). Cyanogen bromide (CNBr)-activated Sepharose powder was washed in a sintered glass filter with 200ml 1mM HCl/ g powder. The protein was added at 5mg protein to 1ml Sepharose and the mixture was rotated overnight at 4°C to facilitate coupling. Excess protein was removed by washing repeatedly with coupling buffer, the first wash being retained and its OD determined at 280nm to assess the efficiency of binding. Unreacted sites on the gel were blocked with 1M ethanolamine pH 9, mixing for one hour at room temperature. The Sepharose gel with attached protein was finally washed alternately with 0.1M sodium acetate/ 0.5M NaCl pH 4, and 0.1M Tris/ 0.5M NaCl pH 8 before pouring columns.

2.8.2. Purification of ovine antibodies

Columns were first flushed with ten volumes of PBS, then pre-eluted with ten

volumes of 0.1M glycine pH 2.5. After a further ten volumes of PBS, samples of heat-inactivated (56°C, 30 minutes) sera were applied to the columns and washed through with running buffer. Bound antibodies were eluted with 0.1M glycine/ HCl pH 2.5; 2ml fractions were collected and neutralised with 325µl 2M Tris per tube. Peak fractions were pooled and the OD at 280nm measured to determine antibody concentration, before the addition of 0.5% BSA to prevent aggregation during storage. Antibody preparations were then dialysed into PBS or RPMI medium before use.

2.8.3. Purification of Rabbit anti-peptide antibodies

Fcγ receptor peptide 1 (see below) was conjugated to CNBr-Sepharose using the above method. Serum from the rabbit primed with this peptide was then passed through the column and the specific anti-peptide antibodies were eluted with 0.1M glycine pH 2.5, neutralised as above.

2.8.4. Affinity Columns for FcR Purification

BSA and sheep IgG were coupled to CNBr-Sepharose as described. 5ml columns were poured and run at 4°C. The columns were first washed with 20 column volumes of PBS, followed by 10 volumes PBS/BSA (0.5%). Pre-elution was carried out with 10 volumes of 0.5M acetic acid in 1% NP-40; then columns were re-equilibrated with 150 volumes 1% NP-40 in PBS.

The two columns were joined and the lysate from 10^8 alveolar macrophages was applied to the BSA pre-column and then to the IgG affinity column. The sample was recycled through both columns overnight. After washing with 150 volumes 1% NP-40 in PBS, the columns were eluted separately using 0.5M acetic acid in 1% NP-40. 1ml fractions were neutralised with 350µl 2M Tris. The fractions were run on SDS-PAGE reducing gels to assess their protein content.

2.9. Proliferation Assays

2.9.1. DC and CD4+ T Cells

2.9.1.1. Response to antigen alone or immune complexes

DC and CD4+ T cells were separated as described above, counted and resuspended in RPMI 1640 containing 10% FCS, 2mM glutamine, 5×10^{-5} M 2-Mercaptoethanol, 100U/ml benzyl penicillin and 100U/ml streptomycin. DC were irradiated with 2500 rads from a caesium 137 source before use in assays. Antigen and antibody dilutions in 50 μ l volumes were added to the plates first, followed by an incubation of 30 minutes at 37°C to allow the formation of immune complexes. The cells were cultured with antigen alone or with antigen and specific antibody in 96 well flat bottomed culture plates (Nunc, Denmark), in a final volume of 200 μ l. In each well, 5×10^4 DC were added to 1×10^5 responding CD4+ T cells.

The controls for these assays included DC or T cells alone, plated with antigen and antibody. After 5 days of culture at 37°C in a humidified atmosphere containing 5% CO₂, plates were pulsed with 1 μ Ci of tritiated methyl thymidine (³H-Thymidine, Amersham) per well and harvested 5 hours later onto filter paper using a semi-automated cell harvester. The incorporation of ³H-Thymidine was assessed by scintillation counting in a beta counter. All of the cultures were performed in triplicate.

2.9.1.2. Inhibition of proliferation

Inhibition of antigen-specific proliferation was assessed by the inclusion of F(ab')₂ fragments of SW73.2, a rat monoclonal specific for ovine MHC Class II (obtained from Dr J Hopkins), throughout the culture period. The control antibody for this experiment was a F(ab')₂ preparation of rat anti-mouse IgG (Pierce, Rockford, Illinois).

2.9.1.3. Inclusion of irrelevant antibody

Anti-HSA antibodies purified from sheep serum on affinity columns as described above (sections 2.8.1. and 2.8.2.) were included in some experiments as an irrelevant control.

2.9.1.4. F(ab')₂ preparations of specific antibody

F(ab')₂ fragments of affinity purified antibodies specific for OVA were used in place of intact antibody in some assays, to assess the importance of the Fc portion of the antibody. Antibody activity against OVA was determined by ELISA before use.

2.9.2. PBMs

Unfractionated PBMs were obtained from defibrinated blood by centrifugation over Lymphoprep. 10⁵ cells per well were cultured with OVA and anti-OVA antibody as described for DC and CD4⁺ T cells. Similar experiments with F(ab')₂ portions of specific antibody, intact irrelevant antibody and anti-MHC Class II antibody were also carried out (section 2.9.1.).

2.10. Fc Receptor Peptides

Peptides were selected from the protein sequence of the extracellular domains of bovine FcγRI (Symons & Clarkson 1992), for their hydrophilicity and likely expression on exposed surfaces of the Fc receptor. Peptide 1 is located in the second, and peptide 2 in the first of the three extracellular domains. Both of the peptides incorporated a cysteine amino acid at the N-terminus.

The peptides were synthesised by Douglas Thomson in the Department of Veterinary Pathology, using standard fluoronylmethoxycarbonyl (Fmoc) chemistry (Dryland & Shepherd 1986). The peptides were desalted on a Sephadex G10 filtration column and

purified by high pressure liquid chromatography (HPLC) using a C₁₈ reversed phase column. Each peptide was freeze-dried and conjugated to OVA at a 1:1 ratio. OVA was reacted with N-succinimidyl 3-[2-pyridyldithio]propionate (SPDP), to provide a thiol group for subsequent formation of a disulphide link with the cysteine residue present at the N-terminus of each of the synthetic peptides. OVA-peptide conjugates were emulsified in Hunter's Titermax, and 100µg of peptide-OVA conjugate was administered subcutaneously in several sites to rabbits.



CHAPTER THREE

PHENOTYPIC ANALYSIS OF DC

3. PHENOTYPIC ANALYSIS OF DC

3.1. Introduction

DC from the afferent lymph of sheep bear surface molecules important in antigen uptake, intercellular adhesion, costimulation and the presentation of peptide antigens (see Chapter 1). Expression of these various molecules contributes to the unique role of DC in the initiation of primary immune responses and the activation of resting T cells. The role of surface molecules such as Igs and Fc receptors for IgG has not been fully investigated.

After intradermal administration of antigen in a primed, cannulated sheep, changes occur in the phenotype and function of afferent lymph DC (Hopkins et al 1989). Surface MHC class II is upregulated, reaching maximum levels five days after challenge. CD1 is upregulated on a proportion of DC after antigen administration. The stimulatory capacity of DC for presentation of an unrelated antigen to T cells *in vitro* increases with elevated class II expression.

In a primary response to antigen no alteration in MHC class II on the cell surface is noted, although CD1 expression is modulated and three different populations of DC emerge transiently with varying intensity of expression. Changes in the expression of surface molecules may enhance the immunostimulatory capacity of afferent lymph DC for the subsequent activation of T cells in the draining lymph node.

In this chapter, phenotypic analysis of resting and *in vivo* or *in vitro* antigen-stimulated DC was carried out. The expression of molecules involved in the uptake and presentation of antigen and intercellular reactions was assessed by flow cytometry.

3.2. DC Phenotype in Resting Lymph

Cells were obtained from pseudoafferent cannulations of the prefemoral ducts of Finnish Landrace or Scottish Blackface sheep as described. Lymph was collected in

sterile bottles containing heparin and penicillin/ streptomycin, and a period of five days was allowed before lymph cells were stained with a panel of monoclonals (details in Table 1, Chapter 2) and analysed by flow cytometry as described in Section 2.4.5. Representative fluorescence histograms are shown in Fig.1.

3.2.1. Presentational Molecules

DC of afferent lymph form a heterogeneous population regarding both morphology and the expression of surface molecules such as CD1 and Fc γ R (Bujdoso et al 1989, Mackay et al 1988a, Harkiss et al 1990). In these experiments, resting DC expressed high levels of MHC class II and class I products, while CD1, as detected by staining with SBU-T6, was expressed by the majority of DC (Fig.1).

3.2.2. Surface Ig

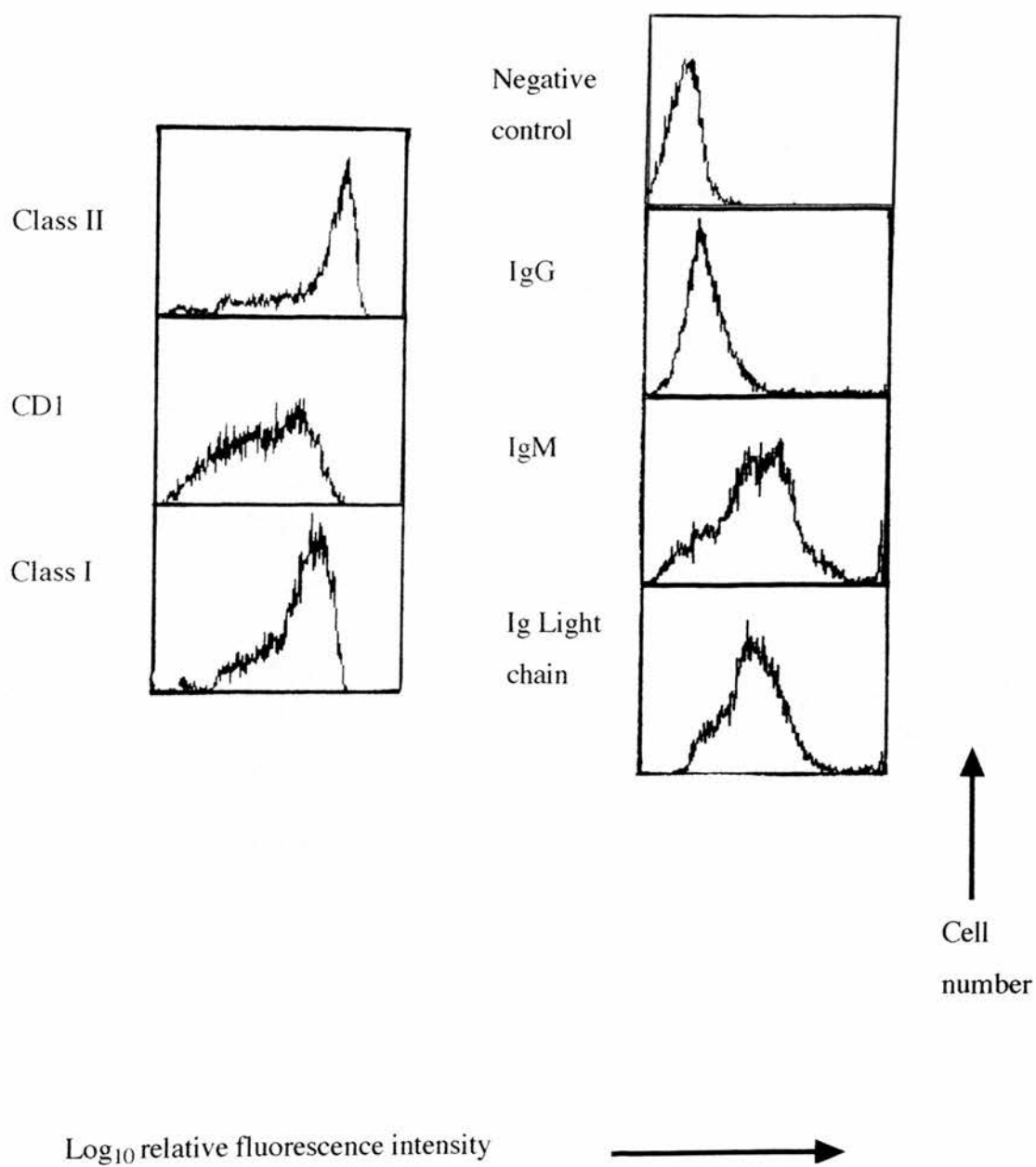
Surface immunoglobulins have been reported on DC from sheep afferent lymph (Miller & Adams 1977, Barfoot et al. 1989). Experiments in this chapter showed that DC staining for surface IgM was variable (Table 1) both between animals and over the time course of a particular cannulation. Addition of lymph to afferent lymph DC expressing low levels of IgM and subsequent incubation at room temperature did not result in IgM binding to DC (data not shown), suggesting that IgM in the lymph is not simply adsorbed onto the DC surface. Staining with VPM8 to detect Ig light chain gave a higher number of DC positive for this marker than for IgM alone, and a small percentage (mean=9%, Table 1) of DC were routinely positive for IgG1. IgG2 and IgA were not detected on DC. Representative flow cytometry profiles are shown in Fig.1.

The percentages of DC positive for each marker were assessed as described in Section 2.4.5.. However, with some surface molecules, including IgG, IgM and CD1, the peaks for the negative control and stained cells overlapped considerably. This made it difficult to set a marker to the right of the negative peak and obtain an accurate percentage of cells positive. For example, the staining profile for IgG in Fig.1 gave 15% DC positive for this molecule when a marker was set to the right of the negative

Figure 1

The cells from afferent lymph were washed and stained with monoclonal antibodies specific for immunoglobulins and molecules involved in the presentation of antigen. The DC were gated on as the cells with high forward and side scatter in the scatter profile of unseparated afferent lymph cells. The negative control peak shows fluorescence where cells were stained with FITC conjugate only. Ten thousand cells were analysed per sample, and representative fluorescence profiles are shown.

FIGURE 1 Flow Cytometry Profiles of Afferent Lymph DC



control peak. However, the whole peak has shifted to the right on staining with anti-IgG mAb, therefore it is arguable that all the cells bear a low level of IgG.

3.2.3. Adhesion/ Costimulation Molecules

Many surface molecules involved in intercellular adhesion and costimulation were expressed by the DC analysed. LFA-1 is thought to be necessary for the formation of DC-T cell clusters from which activated T cells arise (Inaba & Steinman 1986), and is also implicated in costimulation leading to T cell activation. LFA-1 was expressed by DC from sheep afferent lymph, as was LFA-3, the ligand for CD2 on the T cell surface (Fig.2 and Table 1). CD2 was also detected on a proportion of DC; however it has been suggested that this molecule could be passively acquired from T cells rather than synthesised by the DC (Bujdoso et al 1990).

CD44 also plays a role in cellular interactions (Shimizu et al. 1989), and is expressed by many cell types. Increased expression of this molecule occurs on T cell activation, with memory cells expressing CD44 at high levels. MAbs specific for CD44 enhance the CD2-LFA-3-mediated adhesion between T cells and monocytes, and induce monocyte IL-1 production *in vitro* (Haynes et al 1989). CD44 is also important in lymphocyte binding to high endothelial venules for subsequent extravasation (Jalkanen et al 1988). As shown in Table 1, DC staining with anti-CD44 mAb was consistently high. VLA-4, a $\beta 1$ integrin, is present on T cells where it is involved in interactions with ligands in the extracellular matrix as well as intercellular adhesion (Hemler 1990). Binding of VLA-4 to its ligands, VCAM-1 or fibronectin, mediates adhesion and delivers a stimulatory signal to the T cell. VLA-4 was found to be present on afferent lymph DC (Fig.2). VLA-6, another $\beta 1$ integrin found on T cells, was expressed only by a proportion of DC (Table 1, Fig.2). The extracellular matrix ligand for this molecule is laminin. CD29, the $\beta 1$ integrin alpha chain, was present on afferent lymph DC, correlating with the expression of VLA-4 by these cells.

CD4 on helper T cells is necessary for interaction with MHC class II molecules on APCs in the presentation of peptide antigens. CD4 has previously been reported on the

Figure 2

Afferent lymph cells were washed and stained for analysis by flow cytometry as described in Chapter 2. The monoclonal antibodies used are detailed in Table 1, Chapter 2. The DC population was gated on as described, and ten thousand cells were collected per sample. Representative fluorescence profiles are shown. The negative control peak illustrates DC stained with the FITC conjugate only .

FIGURE 2 Phenotypic Analysis of Afferent Lymph DC

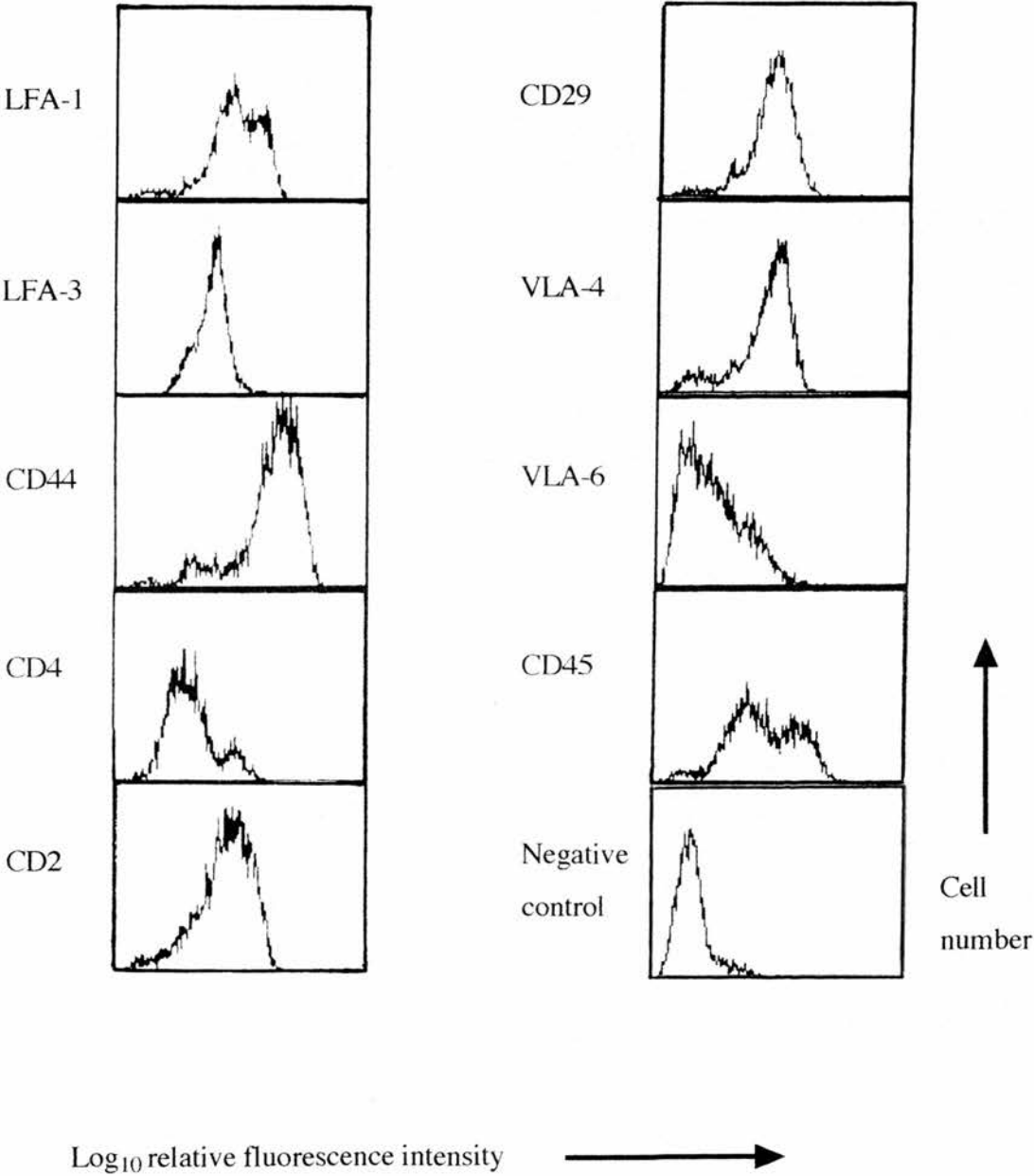


Table 1

Unfractionated afferent lymph cells were collected and stained with monoclonals as described. After application of a FITC-labelled conjugate, cells were analysed for fluorescence expression on a FACScan. DC were gated on as cells with high forward and side scatter: ten thousand cells were analysed per sample. Results are shown as the mean percentage of DC positive for FITC fluorescence over and above the fluorescence of the negative control, with the range in brackets. Analysis was carried out on cells from at least five sheep for each marker; several staining experiments were carried out with each cannulation.

TABLE 1 Phenotypic Analysis of DC From Resting Afferent Lymph

Surface Molecule	DC % Positive for FL-1	
	Mean (Range)	
Class II	95	(80-100)
Class I	97	(91-100)
CD1	78	(61-95)
IgM	50	(7-80)
IgG1	9	(2-23)
Ig Light Chain	51	(12-85)
LFA-1	77	(51-97)
LFA-3	70	(38-95)
CD2	78	(62-88)
CD4	86	(73-92)
VLA-4	81	(62-96)
VLA-6	28	(20-32)
CD29	86	(69-93)
CD44	89	(72-98)
CD45	85	(76-92)

surface of sheep afferent lymph DC (Bujdoso et al 1990). In the phenotypic analysis carried out in this chapter, CD4 was detected on the surface of a variable percentage of the DC population (Table 1), where it may interact with MHC class II on responding cells, providing an activational stimulus for the DC. In the flow cytometry profile shown, a small subpopulation of brightly stained cells is present; these may be contaminating T cells included in the DC gate (Fig.2). CD4-class II interactions will also contribute to cell-cell adhesion (Doyle & Strominger 1987). CD45, the leucocyte common antigen, was also expressed by afferent lymph DC.

3.3. Antigen Uptake

The uptake of antigens by afferent lymph DC has previously been investigated (Harkiss et al. 1990). Enhanced accumulation of fluorescent antigen in the presence of specific antibodies was demonstrated, suggesting the uptake of immune complexes via Fc receptors for IgG on the DC surface.

Here antigen uptake by afferent lymph DC was compared with that by Fc receptor-expressing macrophages. Surface Ig levels on DC were also investigated in antigen uptake experiments.

3.3.1. *In Vitro* Uptake of FITC-OVA

Whole afferent cells were incubated with dilutions of FITC-OVA alone or with lymph from a sheep primed to OVA as a source of anti-OVA antibodies. After incubation for 30 minutes at room temperature, the cells were washed three times and analysed by flow cytometry. 10mM sodium azide was present in the incubation and washing solutions in order to prevent phagocytosis and assess the uptake of immune complexes via FcR. The fluorescence histograms of a representative experiment clearly demonstrate the enhanced uptake of antigen in the presence of specific antibody (Fig.3). At high concentrations of FITC-OVA ($250\mu\text{gml}^{-1}$), over 80% of DCs were positive for FITC fluorescence with added lymph (Fig.3 and Fig.4 (i)). Without lymph, only 40% of DC took up antigen at the same FITC-OVA concentration.

Figure 3

Unfractionated afferent cells were incubated with FITC-OVA at varying concentrations for 30 minutes at room temperature in the presence or absence of lymph from an OVA-primed sheep as a source of specific antibodies. Representative fluorescence histograms are illustrated: the dotted lines show fluorescence of cells incubated in BSA only.

FIGURE 3 DC Uptake of FITC-OVA *In Vitro* is Enhanced by Specific Antibody

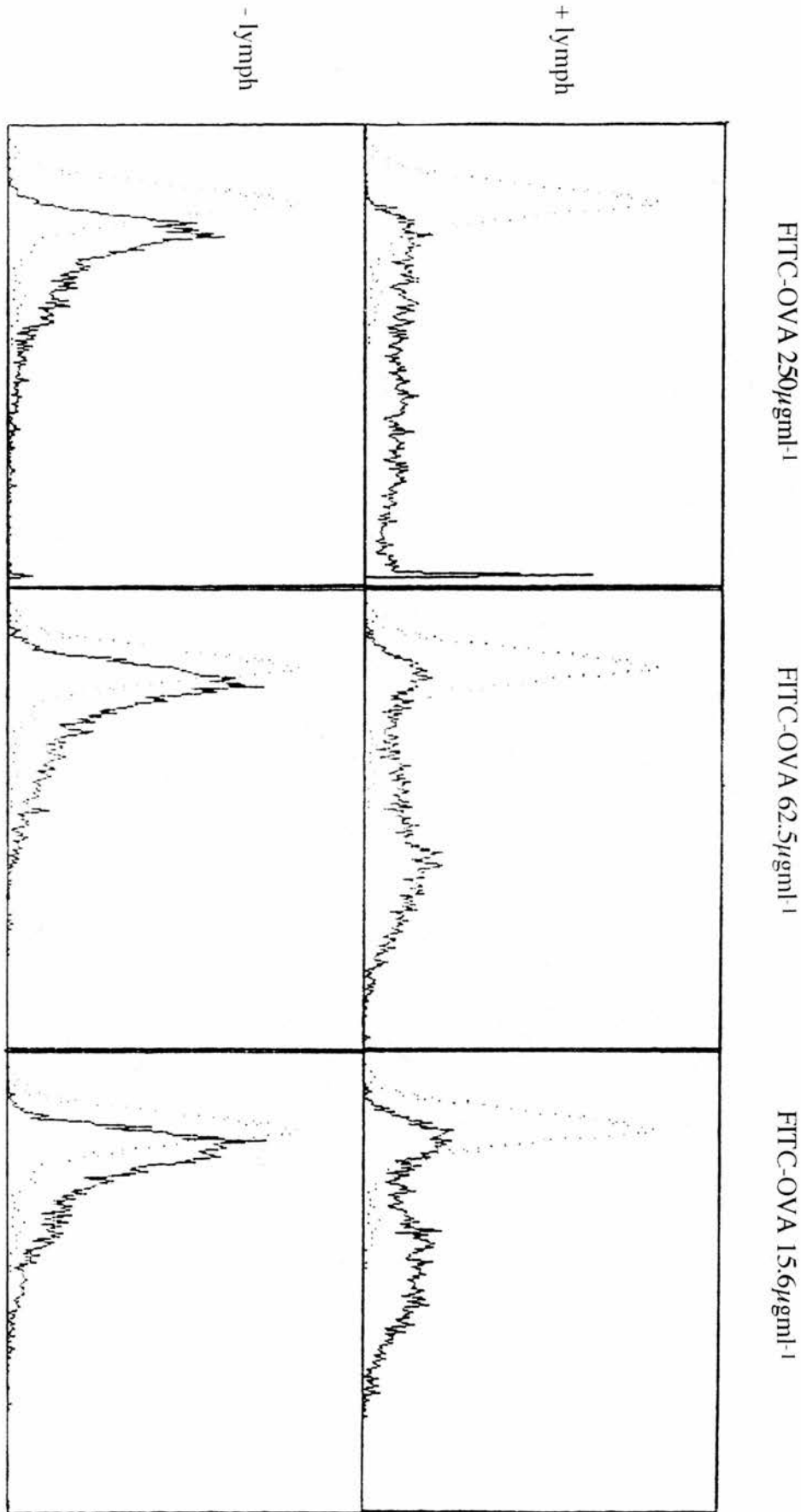
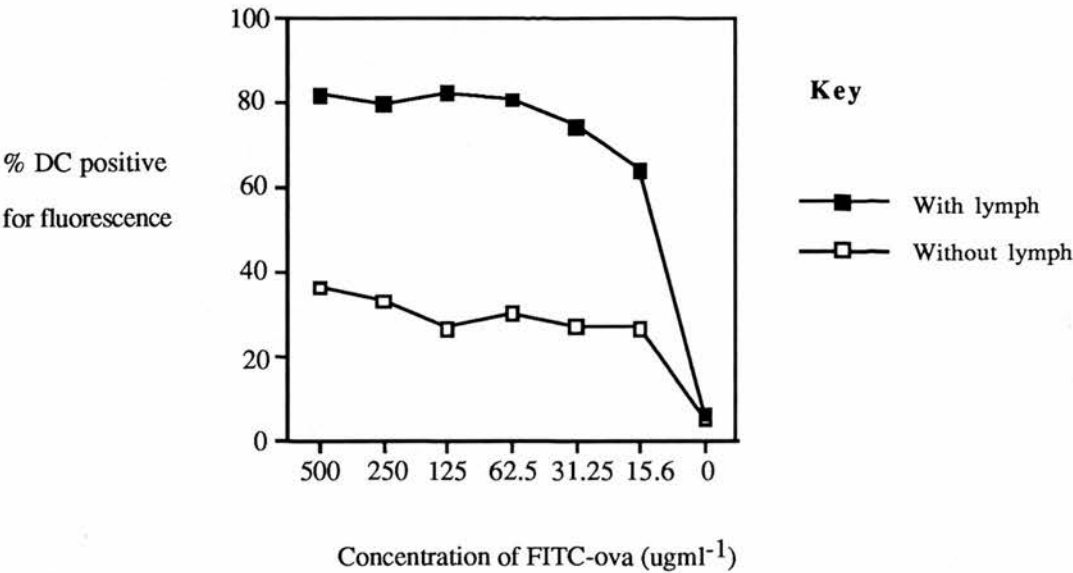


Figure 4

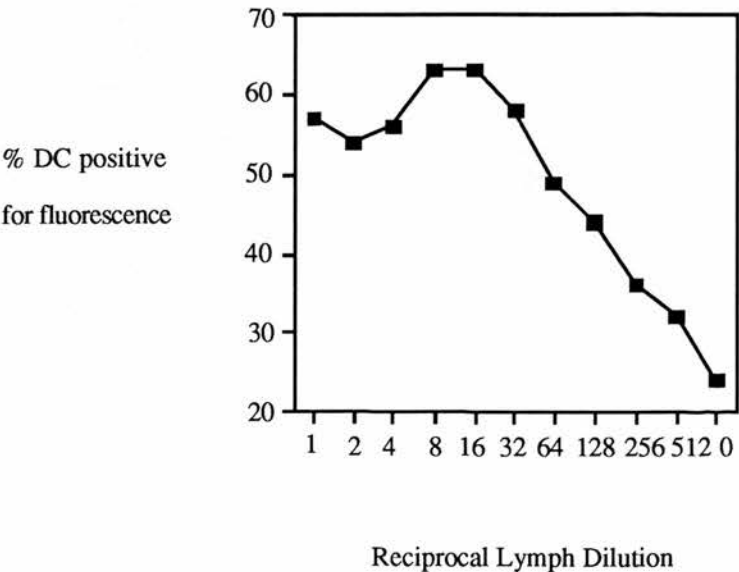
Afferent cells were incubated with FITC-OVA alone, or with lymph as a source of anti-OVA antibodies. The percentage of DC positive for FITC fluorescence is shown. In (i), a lymph dilution of 1/2 was used, with a titration of FITC-OVA. In (ii), serial lymph dilutions were used with a constant FITC-OVA concentration of $25\mu\text{gml}^{-1}$.

FIGURE 4 DC Uptake of FITC-OVA *In Vitro*

(i) Fixed lymph concentration with FITC-OVA dilutions



(ii) Fixed FITC-OVA concentration with lymph dilutions



However, it was difficult to accurately obtain the percentage of DC positive for FITC fluorescence, due to the overlap of cell populations. This was a greater problem with DC incubated with FITC-OVA in the absence of lymph (Fig.3 (ii)), where the peaks representing these cells were clearly shifted to the right, although there was a considerable overlap with the negative control peaks.

In other experiments, a constant concentration of FITC-OVA ($25\mu\text{gml}^{-1}$) and a range of lymph dilutions were used. The percentage of DC showing positive fluorescence varied with the concentration of lymph added, from a minimum of 34% at lymph dilution of 1/512 to 63% at a lymph dilution of 1/8 and 1/16 in the example shown (Fig.4 (ii)). The highest concentrations of lymph used were not the most effective at causing enhanced uptake of antigen. DC did take up some FITC-OVA in the absence of specific antibodies; in this case 24% of DC were positive for FITC fluorescence without added lymph. Lymph alone had no effect on DC fluorescence.

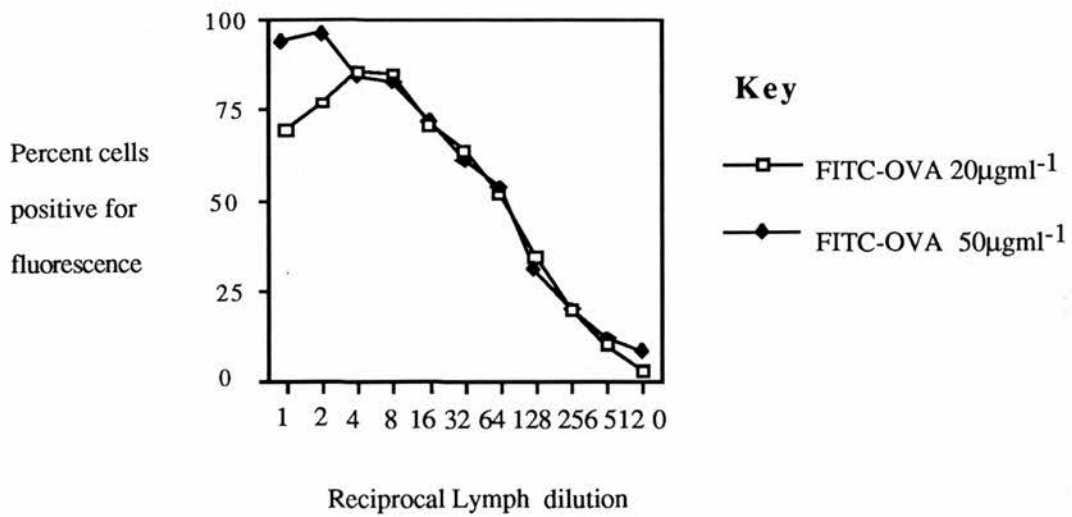
Similar experiments were carried out with alveolar macrophages for comparison. Here either lymph from a primed sheep or affinity purified anti-OVA antibodies were used as a source of specific antibodies, with comparable effects on the enhancement of antigen uptake. Fig.5 shows the percentage of macrophages positive for fluorescence after addition of FITC-OVA at $20\mu\text{gml}^{-1}$ or $50\mu\text{gml}^{-1}$ and either (i) lymph dilutions or (ii) affinity purified antibodies. Only 10% of macrophages took up FITC-OVA alone at $20\mu\text{gml}^{-1}$; when the concentration was increased to $50\mu\text{gml}^{-1}$ only 12% of macrophages were positive for FITC fluorescence. Overlay fluorescence histograms (Fig.6) illustrate the uptake of FITC-OVA in the presence of various lymph dilutions compared with uptake in the absence of lymph. In one experiment, irrelevant affinity purified anti-HSA antibodies were used as a control at the same dilutions as the specific antibody. No increase in fluorescence over that seen with FITC-OVA alone was detected (Fig.5(ii)).

Figure 5

Alveolar macrophages were incubated *in vitro* with FITC-OVA in the presence or absence of specific antibodies, before flow cytometry analysis for detection of FITC fluorescence. In (i), FITC-OVA concentrations of 20 and 50 μ gml⁻¹ were used with lymph from a primed sheep as a source of anti-OVA antibodies. In (ii), affinity purified anti-OVA antibodies were used with FITC-OVA at 20 μ gml⁻¹. Irrelevant affinity purified antibodies specific for HSA were used as a control.

FIGURE 5 Uptake of FITC-OVA by Alveolar Macrophages

(i) Lymph of an OVA-primed sheep as a source of antibodies



(ii) Affinity purified anti-OVA and control antibodies

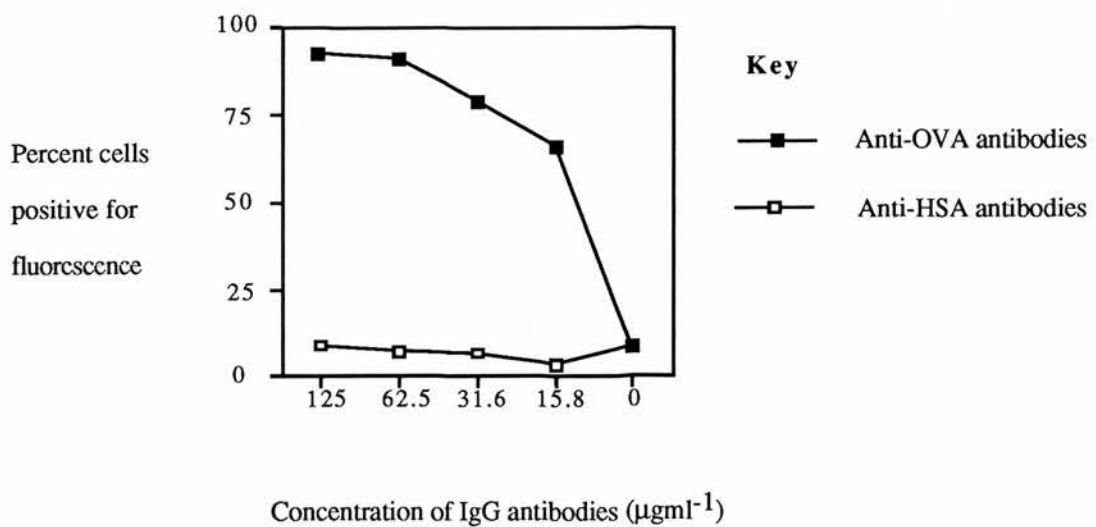


Figure 6

Alveolar macrophages were incubated with FITC-OVA alone at a concentration of $20\mu\text{gml}^{-1}$, or with FITC-OVA and lymph from a primed sheep at various dilutions as a source of anti-OVA antibodies. Cells were analysed by flow cytometry for FITC fluorescence. The solid lines illustrate fluorescence in the presence of FITC-OVA and antibody, while the dotted lines show the fluorescence observed with FITC-OVA only.

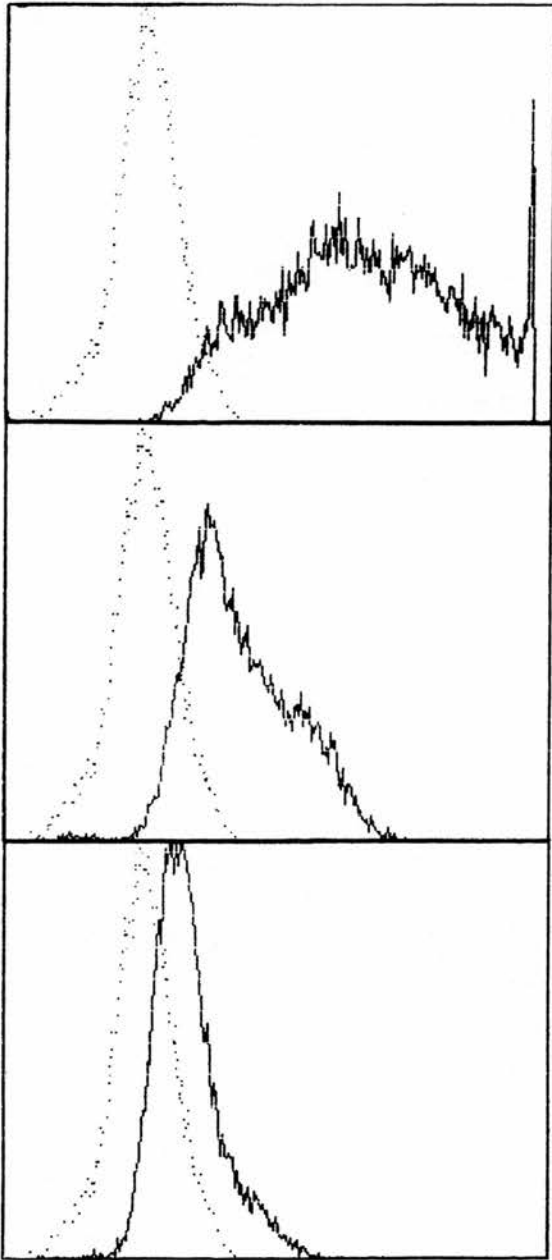
FIGURE 6 Macrophage Uptake of FITC-OVA is Enhanced by Specific Antibody

Dilution of Lymph
containing specific
antibodies

1/1

1/64

1/512



Log₁₀ relative fluorescence intensity

Cell number

3.3.2. DC Staining for Surface Ig After *In Vitro* Antigen Uptake

Assuming that the enhanced uptake described above is due to the accumulation of immune complexes by DC Fc γ R, an increase in the levels of IgG on the DC surface should be detectable. This was investigated using monoclonals specific for Ig isotypes. Unlabelled OVA was used in these experiments as problems were encountered with double staining using PE conjugates to monoclonals and the directly-labelled FITC-OVA. Fig.7 illustrates the increase in sIgG expression detected after *in vitro* incubation of DC with dilutions of OVA and lymph from a primed sheep as a source of antibodies. Surface IgG was detected with both VPM6 and Rsh γ 1, the latter being specific for sheep IgG1. No DC staining was observed with RSh γ 2, specific for sheep IgG2 (Fig.7). A maximum of 34% of DC were positive for sIgG in these experiments. This contrasted with sIgG levels on incubation with BSA only, or with OVA only, which were 4% and 6% of DC positive for fluorescence respectively.

3.3.3. *In Vitro* Uptake with IgM Anti-OVA Antibodies

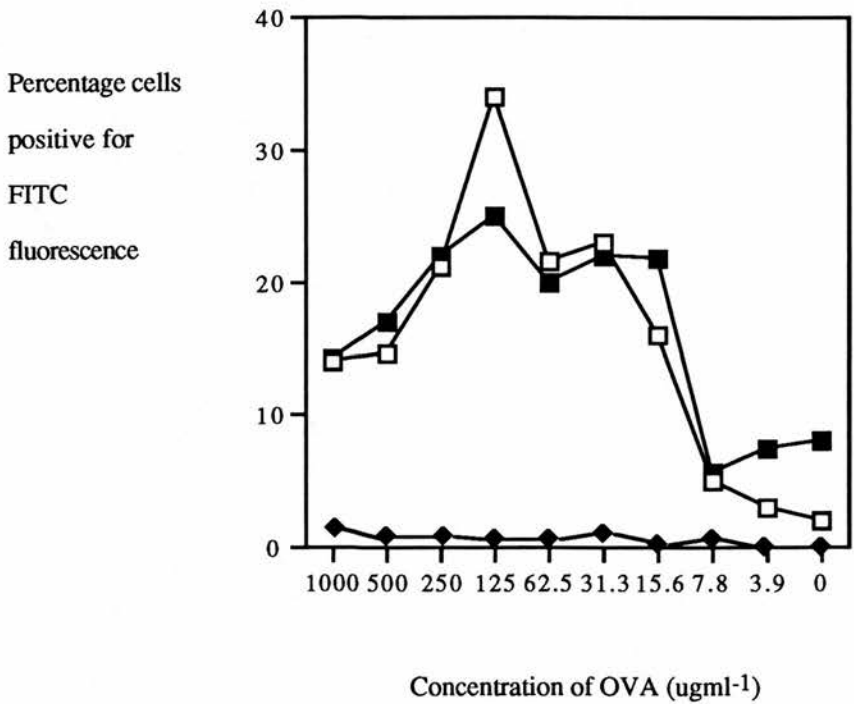
In the primary immune responses for which DC are the crucial accessory cells, specific antibodies of IgG isotype will not be available. Specific IgM antibodies are produced during the primary response, while naturally-occurring antibodies of IgM isotype with multiple specificities may play a role in the initial interaction with antigen (Casali & Notkins 1989). IgM has been detected on the surface of afferent lymph DC, and has been suggested as a concentrating mechanism for antigens in the primary response (Bujdoso et al 1990, Harkiss et al 1990). This possibility was investigated by repeating the antigen uptake experiments described above but substituting anti-OVA antibodies of IgM isotype for those IgG antibodies previously used.

Serum from sheep immunised with OVA on rabbit erythrocytes, as described in Chapter 2, was used as a source of IgM antibodies. Serum samples were collected on days 9, 10 and 11 after initial immunisation. Antibody activity was assessed using ELISAs with OVA-coated plates; IgM anti-OVA antibodies were detected with VPM13, which is specific for ovine IgM, followed by an anti-mouse alkaline phosphatase

Figure 7

Unfractionated afferent lymph cells were incubated with dilutions of OVA, and lymph from an OVA-primed sheep as a source of specific antibodies, for 30 minutes at room temperature. The cells were washed and stained with the anti-IgG monoclonals VPM6, Rsh γ 1, or RSh γ 2, followed by an appropriate FITC conjugate for FACS analysis. The results are the percentage of cells positive for FITC fluorescence.

FIGURE 7 SIgG Levels on DC After *In Vitro* Incubation with OVA and specific antibodies



Key

- IgG1 (RShγ1)
- IgG (VPM6)
- ◆— IgG2 (RShγ2)

conjugate. The IgM anti-OVA titres of the sera used in the uptake experiments were 1/320 and 1/640. No anti-OVA antibody activity of the IgG isotype was detected by ELISA, using VPM6 followed by an anti-mouse alkaline phosphatase conjugate.

There was no increase in the uptake of FITC-OVA by afferent lymph DC on the addition of serum as a source of IgM anti-OVA antibodies. Sera from two different sheep were used at a 1/2 dilution with a range of FITC-OVA concentrations; neither had any effect on antigen uptake by DC. In the same experiment, lymph from a primed sheep containing IgG anti-OVA antibodies was used as a positive control and caused increased uptake of FITC-OVA (Fig.8). Affinity purified IgM antibodies were obtained from the sera of the primed sheep as described in Section 2.8. These antibodies had no effect on the uptake of specific antigen by DC (data not shown).

3.3.4. *In Vivo Uptake of FITC-OVA*

1mg of FITC-OVA in PBS was injected intradermally into the drainage area of a cannulated prefemoral pseudoafferent duct in a primed sheep. Afferent cells were collected at time points following antigen administration and DC were analysed by flow cytometry for fluorescence intensity. As shown in Fig.9, DC took up the antigen with a maximum percentage of cells (90%) positive for FITC fluorescence three hours after antigen administration. By 7 hours after antigen injection only 30% of DC showed positive fluorescence. These results are in agreement with previous studies demonstrating the uptake of FITC-OVA by DC *in vivo* (Harkiss et al 1990).

3.4. DC Phenotype After Antigen Administration

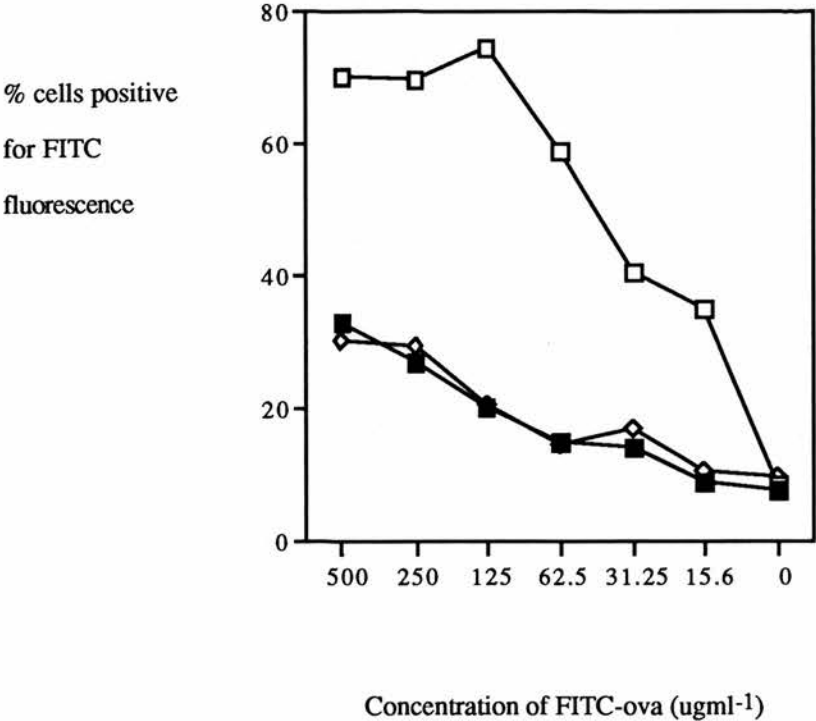
3.4.1. *In Vivo Experiments*

Modulation of DC surface molecules such as MHC class II and CD1 has been reported after *in vivo* challenge with antigen (Hopkins et al 1989). In the following experiments 1mg of FITC-OVA or unlabelled OVA in PBS was injected intradermally into the drainage area of a cannulated lymphatic in a primed sheep. Afferent cells were collected

Figure 8

Unfractionated afferent cells were incubated for 30 minutes at room temperature with FITC-OVA alone, or with added serum or lymph as a source of specific IgM or IgG antibodies. The cells were washed three times in BSA before analysis for FITC fluorescence expression by FACS. The DC were gated on as described.

FIGURE 8 DC Uptake of FITC-OVA is Enhanced with IgG but not IgM antibodies



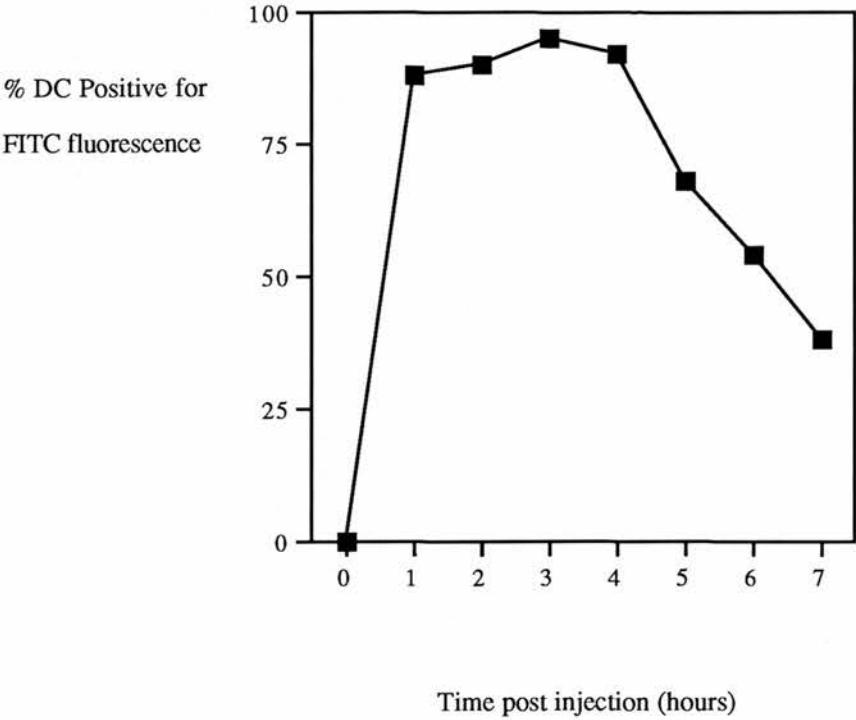
Key

- IgM anti-OVA antibodies
- IgG anti-OVA antibodies
- ◇— No added antibodies

Figure 9

1mg of FITC-OVA in PBS was injected intradermally into the drainage area of a pseudoafferent cannulation in a primed sheep. Afferent lymph cells were collected at various time points after antigen administration and washed before staining with monoclonal antibodies and a PE-labelled conjugate. In this graph fluorescence expression by DC is shown, representing uptake of FITC-OVA *in vivo*.

FIGURE 9 DC Uptake of FITC-OVA *In Vivo* In a Primed Animal



and analysed by flow cytometry at various time points after antigen administration.

Five experiments with injection of unlabelled OVA were carried out, of which four covered a range of time points up to 24 hours after antigen administration. The other experiment included time points of 0 and 24 hours after antigen injection only. In these experiments the cells were stained with monoclonals to surface markers followed by a FITC-labelled conjugate for FACS analysis. A further experiment used FITC-OVA instead of unlabelled OVA; the monoclonals were identified with a PE-labelled anti-mouse Ig conjugate, and afferent cells were collected for analysis over a 7 hour period after antigen administration. Marked changes in the expression of DC surface markers were observed after the intradermal administration of antigen.

3.4.1.1. Surface Immunoglobulins

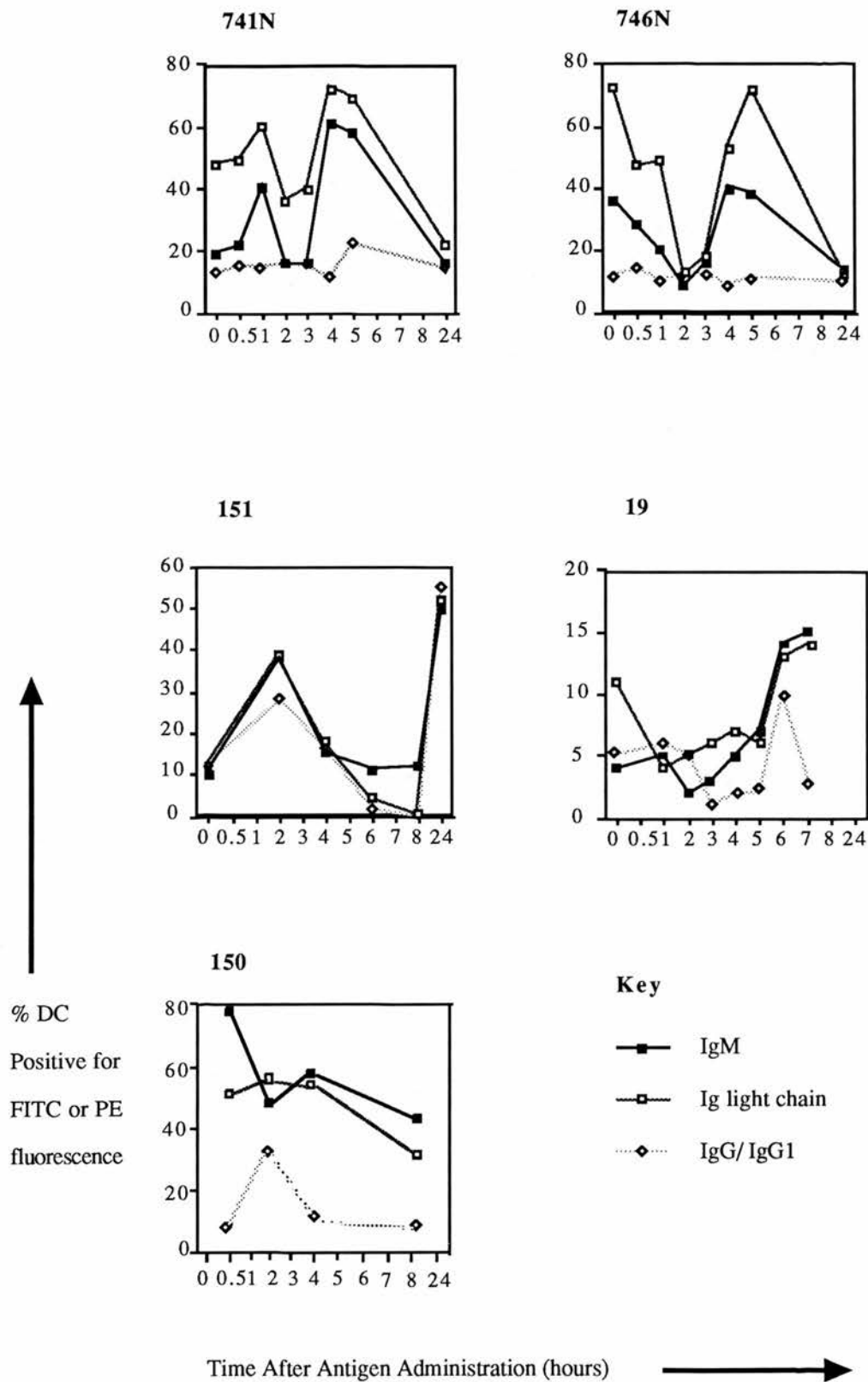
Fig.10 demonstrates DC staining for sIgs in five experiments. The results are difficult to compare directly, as the initial percentage of DC positive for each surface molecule varied between animals, and so are shown separately. Thus DC of sheep 19 showed minimal staining with monoclonals specific for IgM and IgG at the beginning of the experiment although the numbers of cells positive for both, and especially IgM, increased over the seven-hour period monitored. DC from the other four sheep showed considerable variation in immunoglobulin levels, with a transient rise in IgM in two sheep followed by a decrease to around initial levels. Sheep 746N showed a dramatic decrease in staining with VPM13 and VPM8 by 2 hours, followed by an increase in both markers. There was thus an increase in the percentage of DC positive for IgM at some point after antigen administration in all of the sheep except 150. DC from the latter showed a decrease in the percentage of cells positive for IgM by 2 hours after antigen injection which was maintained over the time course of the experiment.

In two of the five sheep (150 and 151), the number of DC bearing IgG increased over the first two hours after antigen injection (Fig.10). The increase was from 9 to 33 % of DC showing positive staining for fluorescence in sheep 150, with a corresponding 11 to 28% increase in DC from sheep 151. This increase in IgG levels may be due to the

Figure 10

1mg of OVA (or FITC-OVA in sheep 19) was injected intradermally into the drainage area of pseudoafferent cannulations in primed sheep. Afferent cells were collected at various time points after antigen administration and stained with monoclonals specific for sheep Igs for FACS analysis. In sheep 741N, 746N, 151 and 150, a FITC-labelled conjugate was used while in sheep 19 a PE conjugate was the second layer reagent. DC were gated on as cells with high forward and side scatter; 10,000 cells were analysed for FITC or PE fluorescence per sample. All experiments except for that with sheep 19 were carried out over a 24 hour period.

FIGURE 10 Changes in DC Phenotype After Antigen Administration *In Vivo*



presence of immune complexes containing IgG-anti-OVA antibodies attached to the DC surface.

By 24 hours after antigen administration, three sheep showed numbers of DC staining for sIg similar to the initial percentages, while a substantial increase in the number of DC bearing both IgM and IgG was seen with sheep 151. There was therefore a transient decrease in the percentage of DC staining for both IgM and Ig light chain in all of the 5 sheep used. This drop occurred at around 2 hours after antigen administration in 4 sheep but not until 6-8 hours after injection of OVA in the remaining animal. Flow cytometry histograms showing DC staining for IgM at various time points in the experiment with sheep 151 are illustrated in Fig.13.

3.4.1.2. Class II and CD1

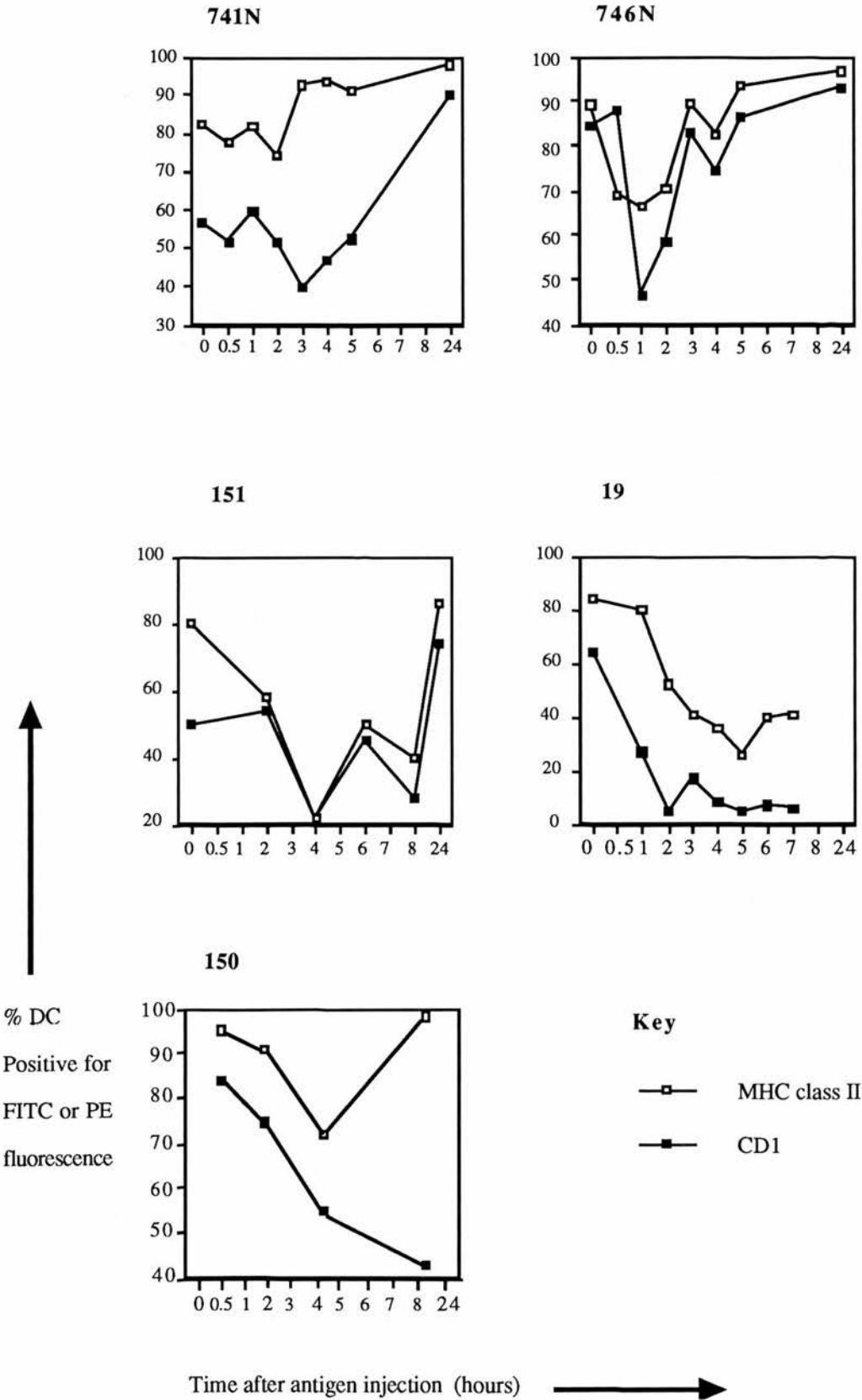
All of the sheep showed an initial decrease in the percentage of DC expressing class II and CD1 molecules (Fig 11). Sheep 151 showed the most dramatic transient decrease, with a fall from 80% to 24% DC positive for class II at the four hour time point, while the percentage of CD1+ cells decreased from an initial 50% to 22% of DC at the same time point. A gradual increase in the expression of both markers then occurred, with a higher percentage of DC positive for both class II and CD1 24 hours after antigen injection. In the experiment with sheep 150, the number of CD1+ DC decreased steadily throughout the experiment, and had not returned to initial levels by the 24 hour time point.

A substantial decrease in the percentage of DC expressing both MHC class II and CD1 molecules therefore occurs, between 1 and 4 hours after antigen administration *in vivo*. Recovery to initial levels takes place over a variable time period between sheep; in some cases the number of cells positive is still below the initial level by 24 hours, while in others the percentage of DC expressing CD1 or MHC class II has returned to initial levels by around 4 hours after antigen injection. The kinetics of the alterations may be affected by the exact site of antigen administration and the number of sites used for each animal. Antigen was injected intradermally into the flank drainage area of the cannulated

Figure 11

Afferent cells were collected at time points after the intradermal injection of 1mg OVA in five primed sheep. The experiments are as described in the legend of Fig.10. The monoclonal antibodies used were SBU-T6 for CD1 and VPM36 for MHC class II.

FIGURE 11 MHC Class II and CD1 Expression by DC After Antigen Administration *In Vivo*



prefemoral duct, usually in two or three separate sites, but these were not necessarily the same in each case. Flow cytometry profiles for CD1 and class II staining on the afferent lymph DC of sheep 151 are shown in Fig.12, and illustrate the marked differences in both the proportion of cells positive for these molecules, and the alteration in their levels of expression by DC at various time points.

3.4.1.3. Adhesion/ Costimulation Molecules

DC expression of adhesion molecules was investigated in three sheep. One experiment, with sheep 150, followed changes in the expression of adhesion molecules over a 24 hour period, while another two experiments using sheep 151 and sheep 147 included 0 and 24 hour time points only. In addition, CD44 levels on DC from sheep 151 were measured at several points over a 24 hour time scale (Fig.13).

The percentage of DC staining for CD44 remained constant over the 24 hour time course with sheep 150 (Fig.14) and sheep 151 (Fig.13), although a decrease in the levels of CD44 expression did occur in both cases, as shown by the sequential fluorescence profiles. In the experiment with sheep 147 where only a 24 hour time point was taken, CD44 staining at 24 hours was slightly above the initial percentage (Fig.15).

LFA-1 expression by DC from sheep 150 was fairly constant over the first four hours but had decreased to only 37% of DC (compared with an initial 76%) by the 24 hour time point (Fig.14). DC of Sheep 147 showed a slight increase in expression of LFA-1 at 24 hours, while in sheep 151 the percentage of DC expressing LFA-1 had increased from an initial 25% to 62% at 24 hours (Fig.15).

The percentage of DC positive for LFA-3 in sheep 150 had decreased four hours after the administration of antigen, and levels were still low compared with initial expression by the 24 hour time point (Fig.14). In agreement with this, LFA-3 was present on only 40% of DC at the 24 hour time point in sheep 147 compared to 82% before antigen injection. However, in the experiment with sheep 151, LFA-3 expression increased

Figure 12

1mg of OVA was injected intradermally in a primed sheep (Number 151). The afferent cells were collected at various time points after antigen administration, and stained for flow cytometry analysis. Fluorescence histograms for DC are shown for CD1 and MHC class II expression . The faint lines show the negative control peaks representing staining with VPM53.

FIGURE 12 DC Expression of Surface Molecules After Antigen Administration *In Vivo*

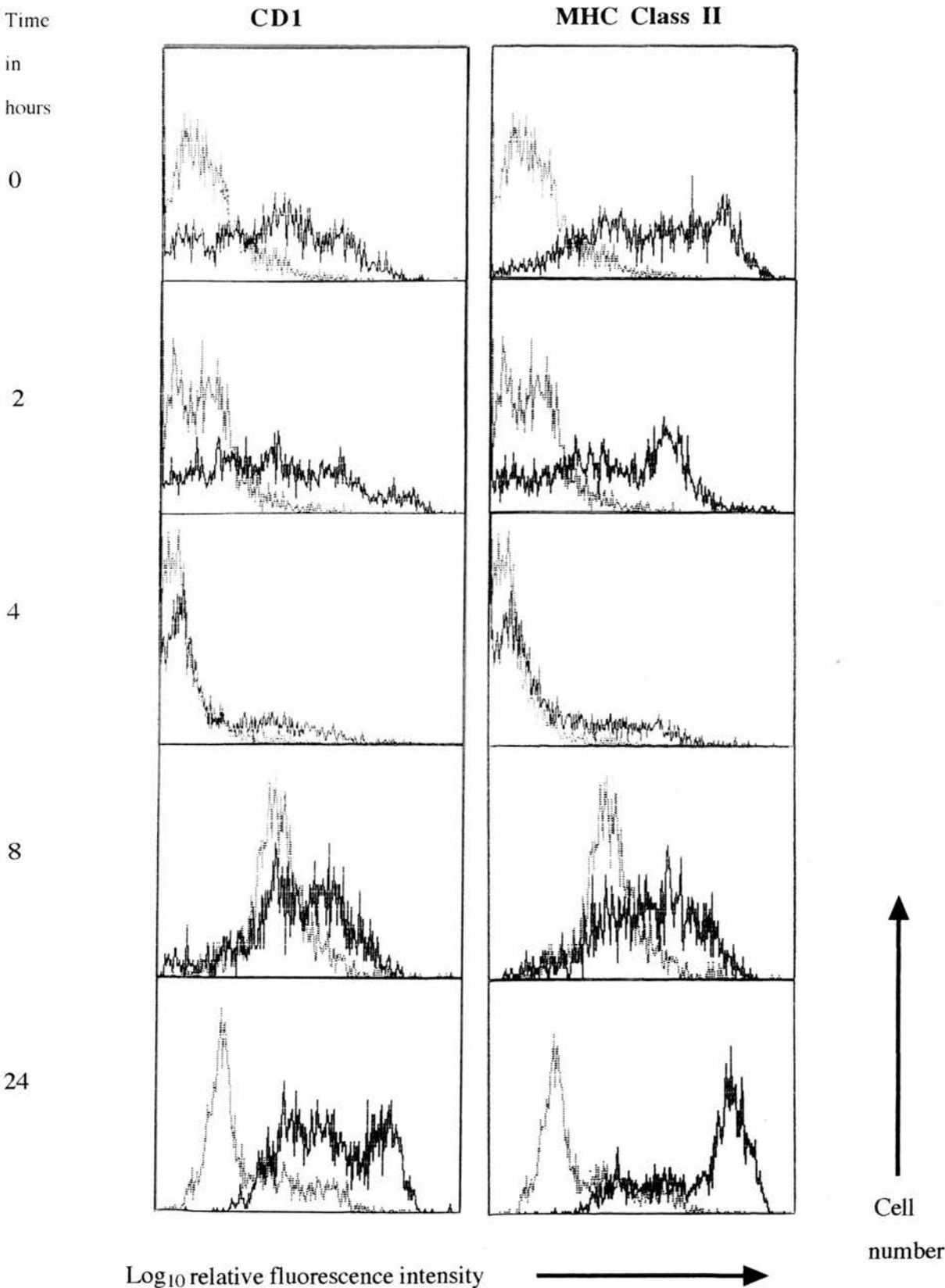


Figure 13

Afferent cells were collected and prepared for flow cytometry analysis at various time points after administration of 1mg OVA into the drainage area of a cannulated, primed sheep. Cells were stained with MAbs specific for CD44 and IgM. DC were gated on as described. The faint lines show the negative control peaks representing staining with VPM53.

FIGURE 13 DC Phenotype After Antigen Administration *In Vivo*

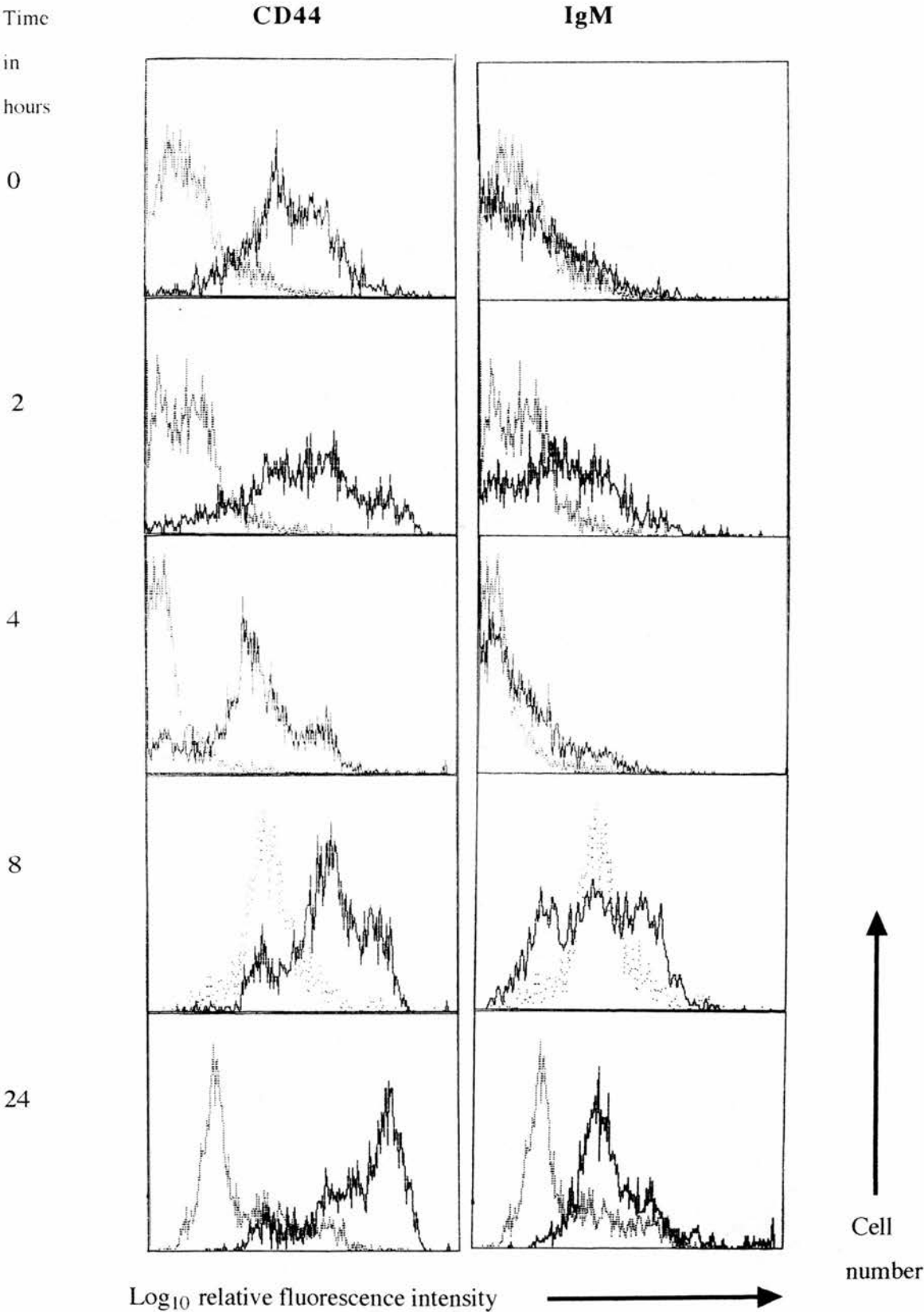


Figure 14

1mg of OVA was injected into the drainage area of a cannulated, primed sheep (150). Cells were collected at 2,4 and 24 hour time points following antigen administration, and stained for flow cytometry analysis with mAbs specific for CD44, LFA-1 and LFA-3. DC were gated on as described, and negative control peaks (faint lines), representing staining with VPM53, are overlaid.

FIGURE 14 Flow Cytometry Profiles of DC At Various Time Points After Antigen Administration *in vivo*

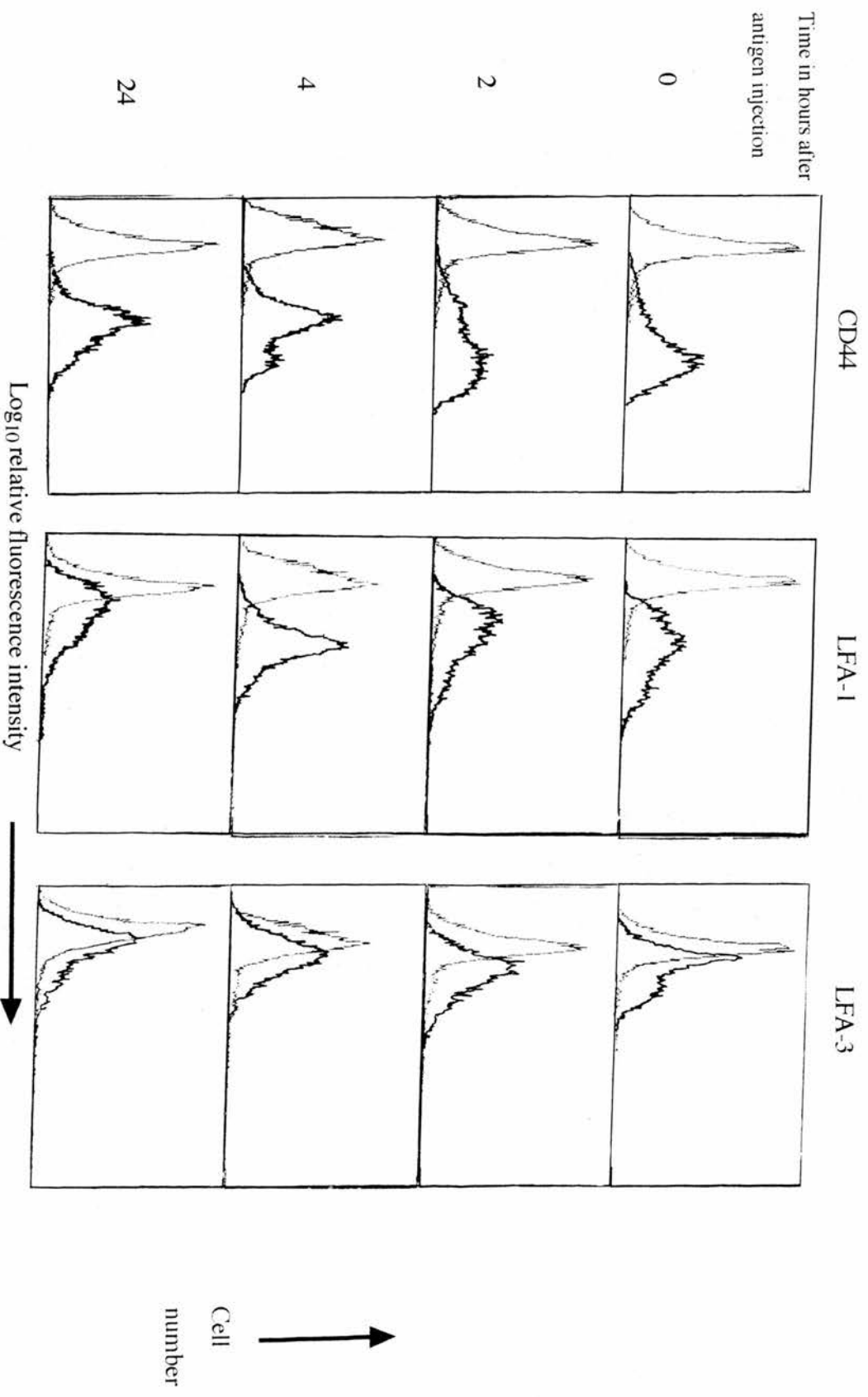
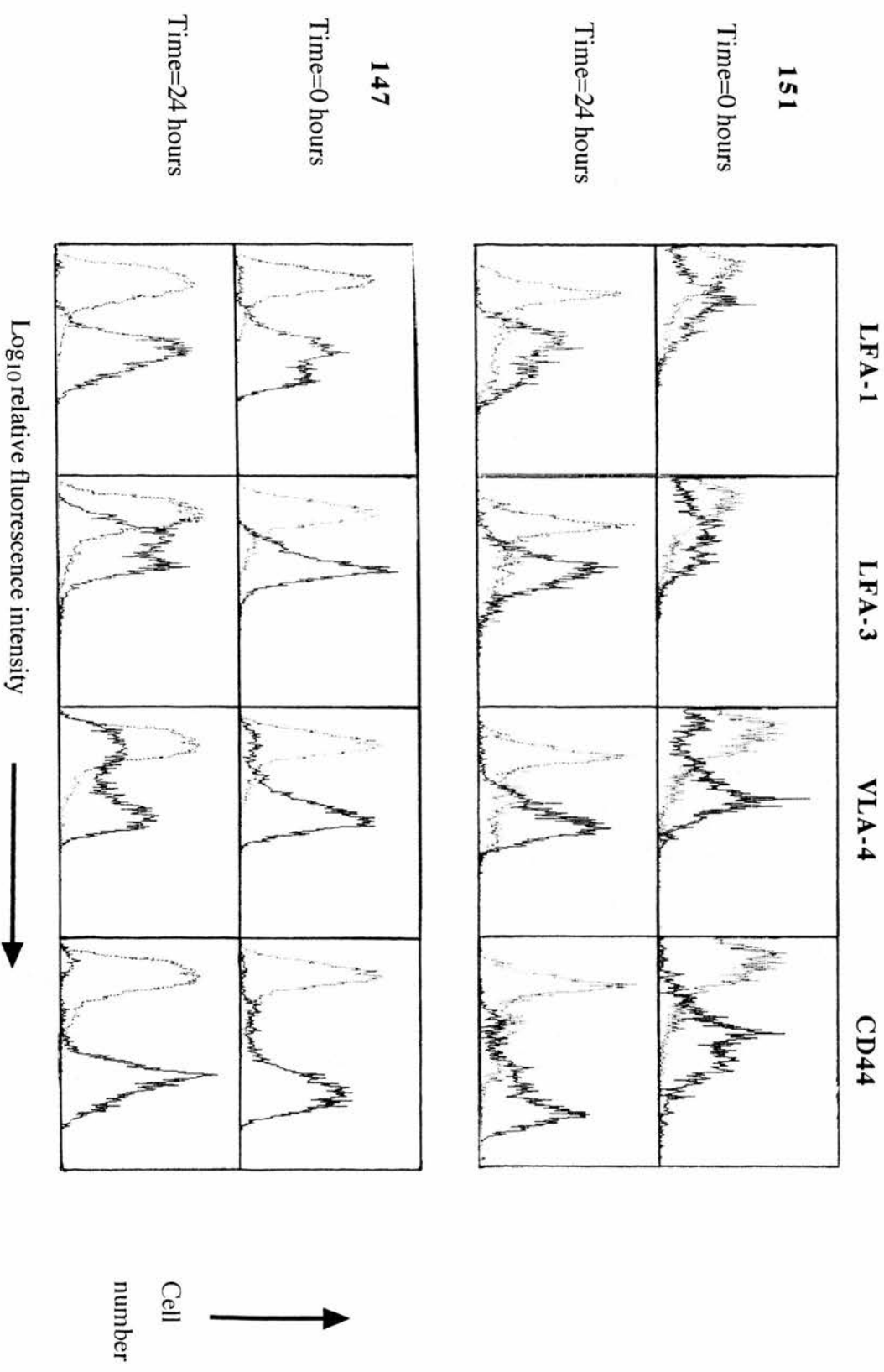


Figure 15

1mg OVA in PBS was administered intradermally into the drainage area of a cannulated prefemoral pseudoafferent lymphatic, in each of two primed sheep. Lymph was collected prior to antigen injection and 24 hours later. Cells were prepared for FACS analysis, using MAbs specific for LFA-1, LFA-3, VLA-4 and CD44, and DC were gated on as described. Staining profiles (solid lines) are overlaid with the negative control peaks for each time point which represent staining with an isotype-matched irrelevant MAb, VPM53 (dotted lines).

FIGURE 15 FACS Analysis of DC After OVA Administration to Primed Sheep



from 28% at the start of the experiment to 55% of DC 24 hours after the administration of antigen (Fig.15). These results appear to be contradictory, but may reflect similar changes in expression to those seen with CD1 and MHC class II, but with differences between animals in the time scale over which variation takes place.

A decrease in DC staining for VLA-4 occurred in the experiment with sheep 147, from an initial 76% of DC positive to only 46% at 24 hours. In contrast, VLA-4 was detected on a greater proportion of DC at 24 hours in the experiment with sheep 151 (Fig.15). These changes may reflect a decrease and subsequent increase in the percentage of DC expressing these molecules; the contradictory results between sheep may be attributable to a different time scale over which changes occur. A full 24 hour experiment with analysis of DC for VLA-4 expression at several time points would be required to confirm this.

3.4.2. *In Vitro* Experiments

DC expression of surface markers was also examined after antigen administration *in vitro*. In this experiment, unfractionated afferent lymph cells from an OVA-primed sheep were cultured at a concentration of 10^6ml^{-1} in 24 well plates. The culture was carried out in medium alone, OVA at $1\mu\text{gml}^{-1}$, or OVA at $1\mu\text{gml}^{-1}$ and specific antibodies at $30\mu\text{gml}^{-1}$. Flow cytometry analysis was performed before the culture, and after 2 and 8 hours incubation at 37°C . The viability of cells from each group was analysed at each time point by Trypan Blue exclusion and was $>99\%$ in each case.

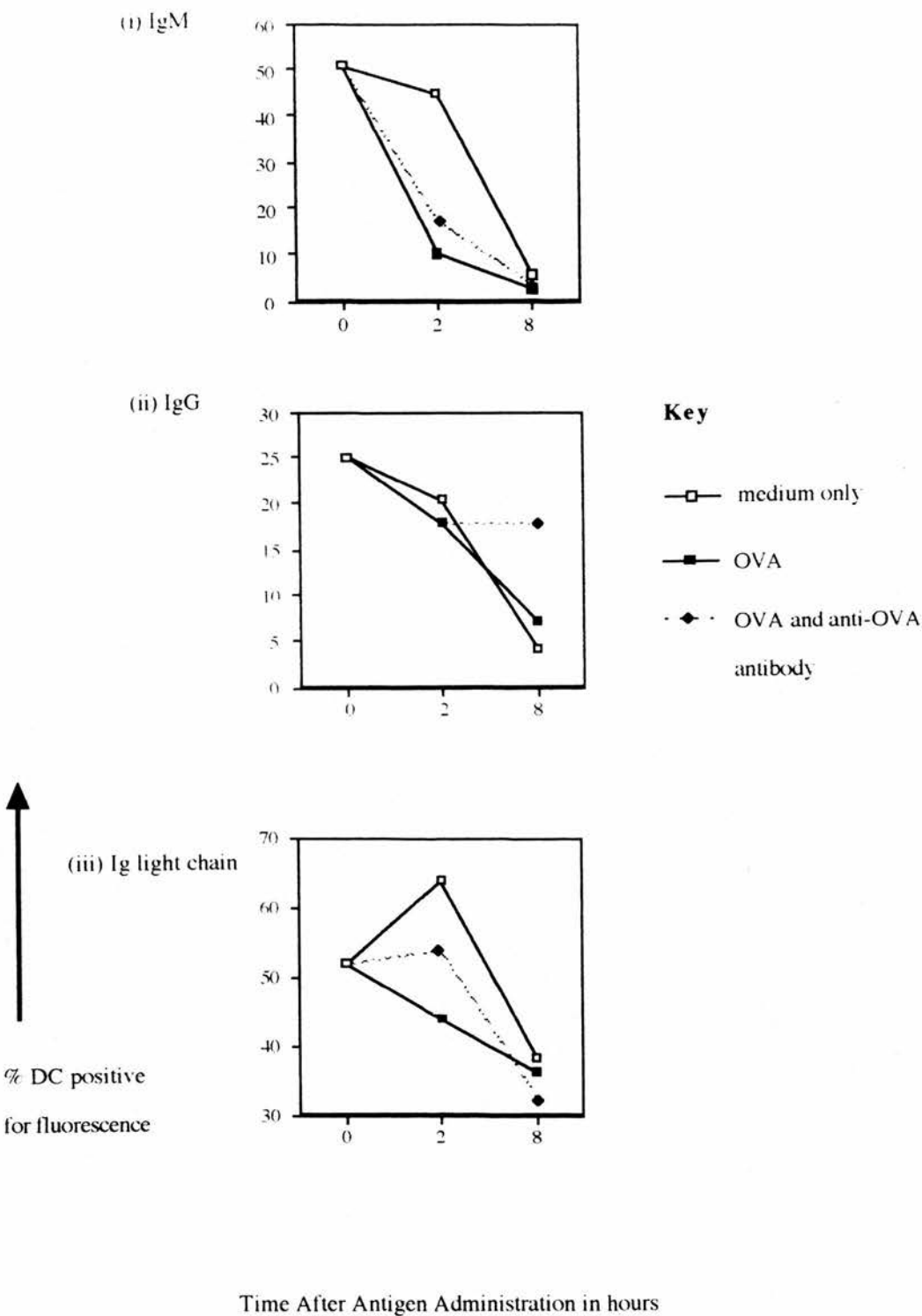
3.4.2.1. Surface Ig

Staining for all of the surface Igs investigated decreased over the culture period where afferent cells were cultured in medium alone (Fig.16). Initially 56% of DC were positive for IgM; on culture in medium only this decreased to only 7% after 8 hours of culture. A more rapid decrease in the percentage of DC staining for IgM occurred where cells were cultured with OVA, or OVA and specific antibody, but there was no

Figure 16

Afferent lymph cells from an OVA-primed sheep (number 151) were cultured *in vitro* in medium alone, OVA at $1\mu\text{gml}^{-1}$, or OVA at $1\mu\text{gml}^{-1}$ and anti-OVA antibodies at $30\mu\text{gml}^{-1}$ (see key). The cells were harvested, washed and stained with monoclonal antibodies specific for Igs; VPM8 for Ig light chain, VPM13 for IgM and VPM6 for IgG. The viability of the cells was assessed for each culture by Trypan Blue exclusion. DC were gated on as described.

FIGURE 16 DC Staining For SIg After *In Vitro* Culture



difference between these two groups. Fluorescence profiles of DC staining for IgM are shown in Fig.17.

The number of DC positive for IgG as detected with VPM6 dropped from 25% to 4% at the 8 hour time point for cells cultured with medium only (Fig.16). When cells were cultured with antigen, a similar decrease took place. In contrast, DC cultured with antigen and antibody showed an initial decrease in expression of IgG at 2 hours of culture, followed by a maintenance of this level to 8 hours after antigen administration. This retention of IgG on the DC membrane is probably due to the presence of IgG anti-OVA antibodies in immune complexes bound to DC Fc γ R. Ig light chain expression over the culture period resembles that of IgM (Fig.16), probably because more IgM than IgG is present on the DC surface.

3.4.2.2. Class II and CD1 Expression

Over the eight hour culture period, the expression of MHC class II molecules by DC remained more or less constant when the cells were cultured with medium or OVA. However, on incubation with OVA and specific antibody at these concentrations, MHC class II expression decreased from 95% to 70% of DC (Fig 18).

A similar result is seen with CD1 expression (Fig.18). The percentage of DC positive for CD1 as measured by staining with SBU-T6 decreased slightly on culture with OVA or medium, but a much greater fall was seen after an 8 hour incubation with OVA and specific antibody. The dramatic fall in the proportion of afferent lymph DC expressing CD1 and MHC class II after *in vitro* culture with OVA and anti-OVA antibody is similar to that which occurs *in vivo* after antigen administration in a primed sheep (3.4.1.2.). Fluorescence histograms for CD1 and MHC class II staining of DC on *in vitro* culture are shown in Fig.19.

3.4.2.3. Adhesion/ Costimulation Molecules

The proportion of DC positive for CD44 remained fairly constant for those incubated

Figure 17

Afferent lymph cells were cultured *in vitro* with medium only, OVA at $1\mu\text{gml}^{-1}$, or OVA at $1\mu\text{gml}^{-1}$ and anti-OVA antibody at a concentration of $30\mu\text{gml}^{-1}$. Cells were stained for flow cytometry analysis with VPM13, specific for ovine IgM, at 0, 2 and 8 hour time points; DC were gated on as described previously. Staining with VPM13 is shown by the solid lines, while the dotted lines illustrate staining with VPM53, an irrelevant isotype-matched MAb.

FIGURE 17 Flow Cytometry Profiles of DC Staining For Surface IgM After *In Vitro* Culture

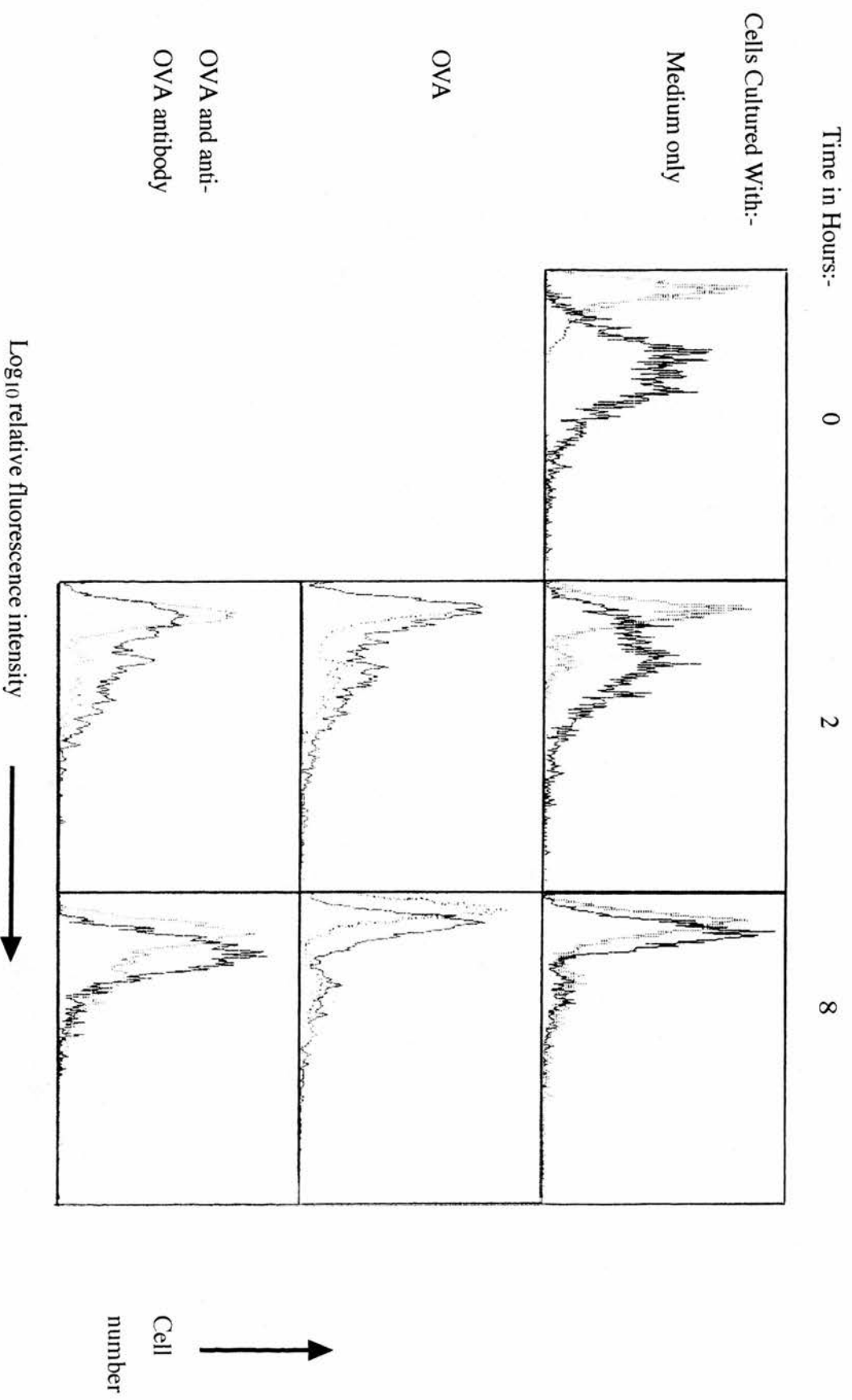


Figure 18

Afferent lymph cells were cultured at 37°C with medium only, OVA at 1µgml⁻¹, or OVA at 1µgml⁻¹ with anti-OVA antibodies at 30µgml⁻¹. The cells were harvested at 2 and 8 hour time points and stained with VPM36 for MHC class II and SBU-T6 for CD1, followed by a FITC conjugate. The DC were analysed by FACS for expression of FITC fluorescence.

FIGURE 18 DC Expression of MHC Class II and CD1 After *In Vitro* Culture

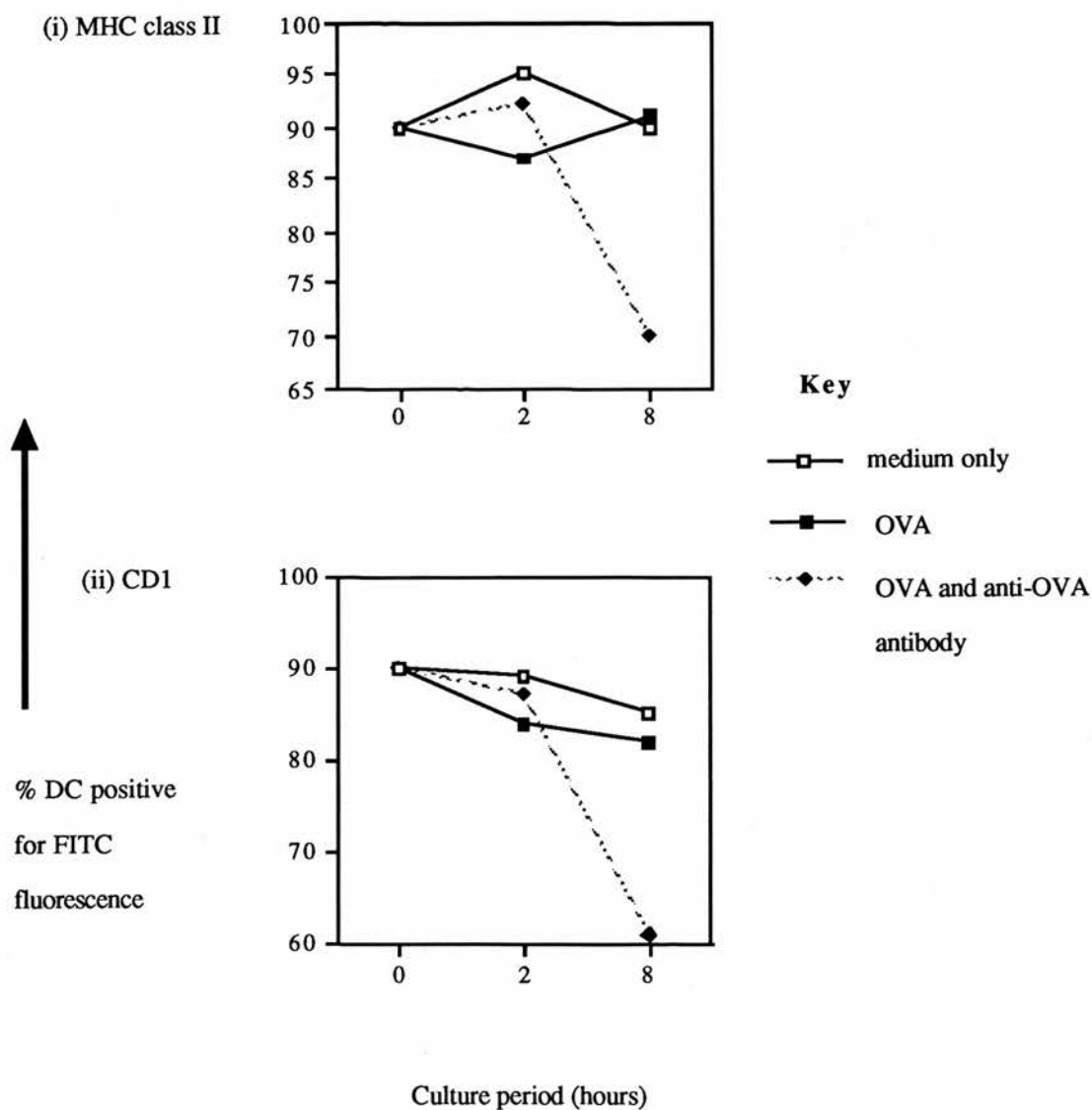


Figure 19

Afferent lymph cells were stained for flow cytometry analysis after *in vitro* culture with:- (i) VPM36 to detect MHC class II expression and (ii) SBU-T6, specific for CD1. DC were gated on according to their light scatter characteristics. Negative control histograms are overlaid (dotted lines).

FIGURE 19 (i) Flow Cytometry Profiles of DC After *In Vitro* Culture: MHC Class II

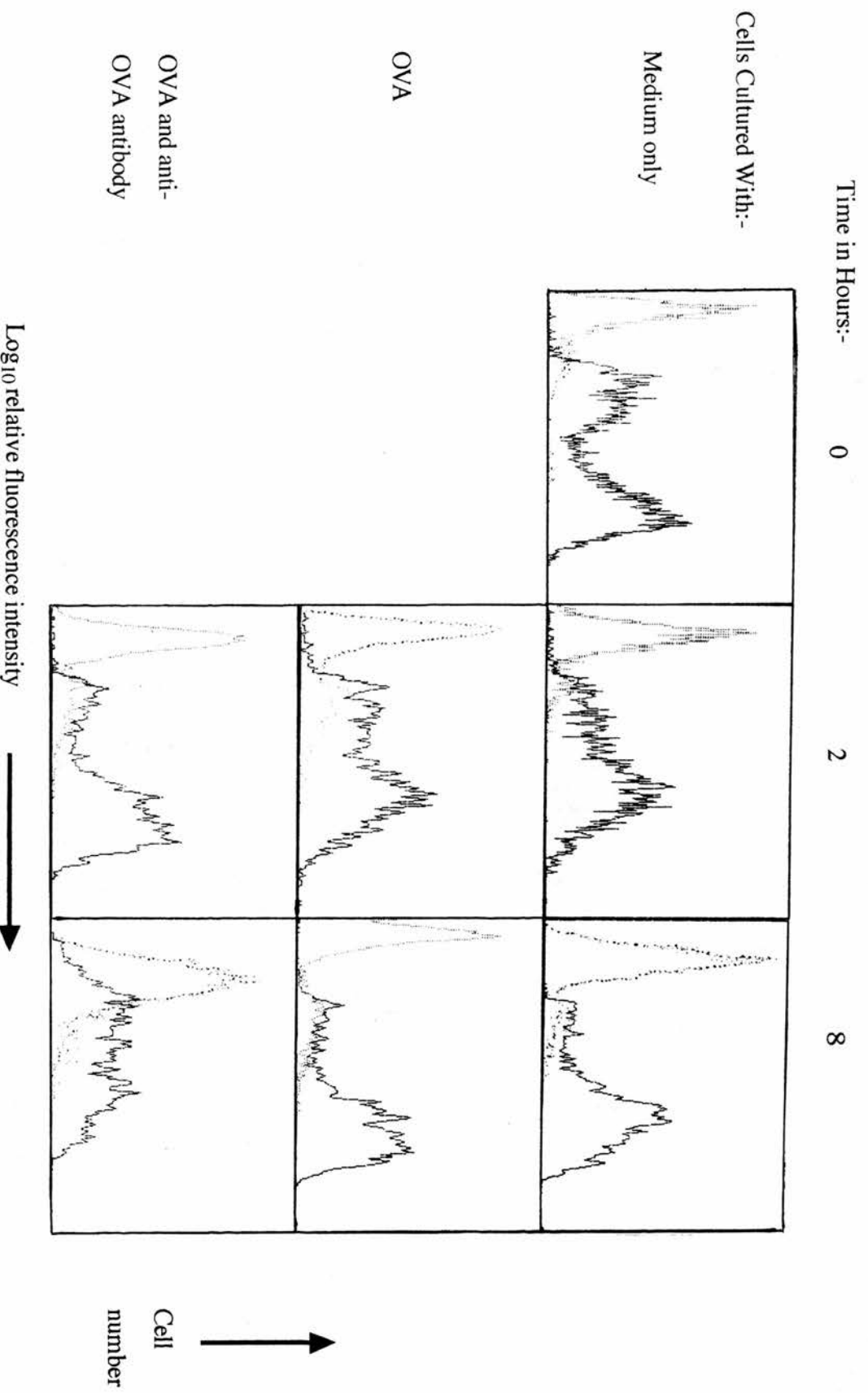
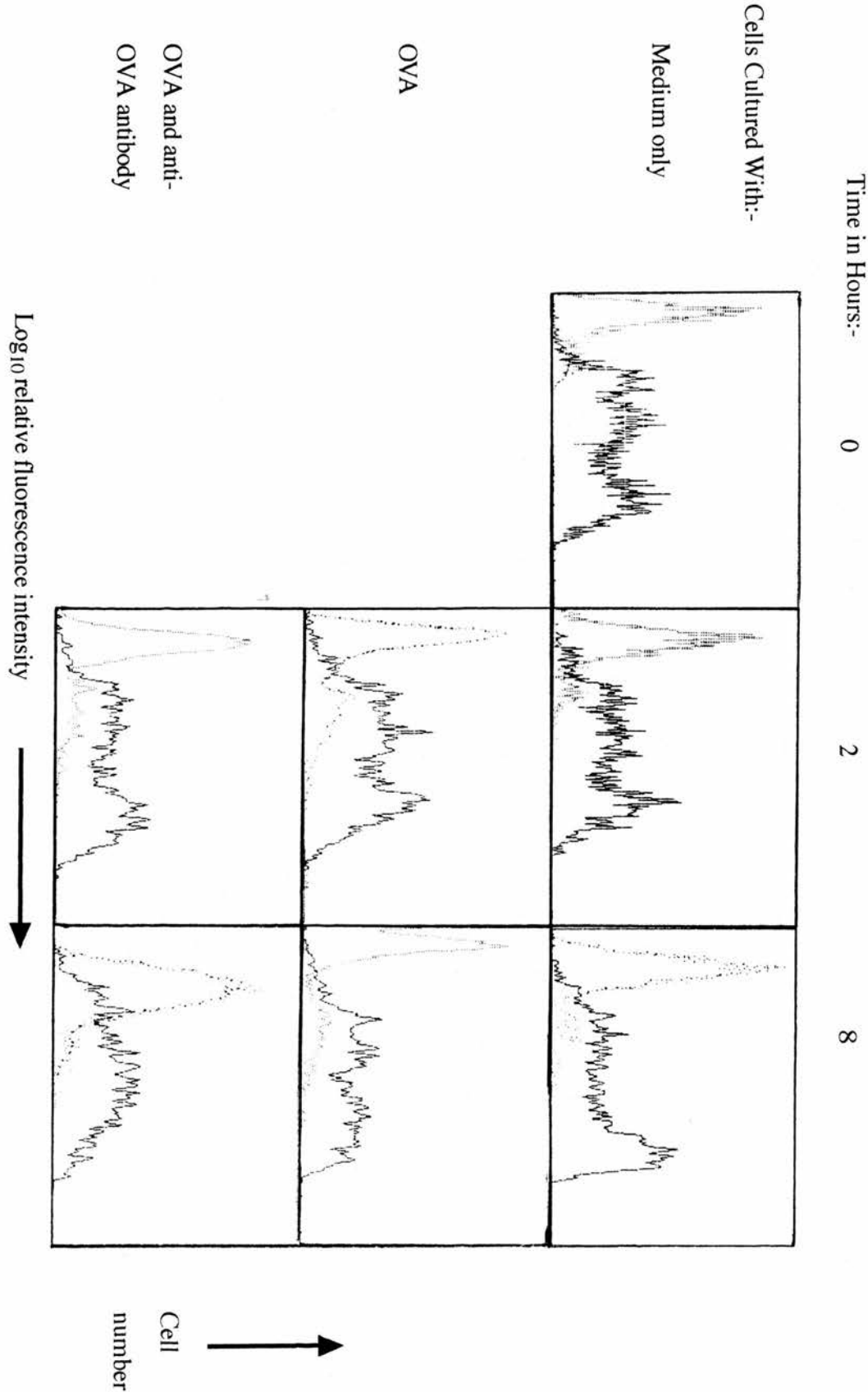


FIGURE 19 (ii) Flow Cytometry Profiles of DC After *In Vitro* Culture: CD1



with OVA or medium, while levels decreased substantially, from 98% to 76% of cells positive, after eight hours of culture in the presence of OVA and anti-OVA antibodies (Fig.20 and Fig.21). A similar change was observed in the expression of VLA-4 on DC, with little variation where cells were cultured with either medium or OVA alone, but a decrease from 97% to 62% of DC positive for VLA-4 after an eight hour culture period with OVA and specific antibody (Fig.20). The number of DC positive for LFA-1 and LFA-3 decreased on incubation for all of the culture conditions (Fig.20). However, in both cases the greatest drop was seen where cells were incubated with antigen and specific antibody. The alterations in the levels of these adhesion molecules on afferent lymph DC after *in vitro* culture with OVA and anti-OVA antibody are similar to the changes observed in the expression of MHC class II, CD1 and IgM on DC under the same conditions.

3.7. Discussion

3.7.1. Resting Phenotype

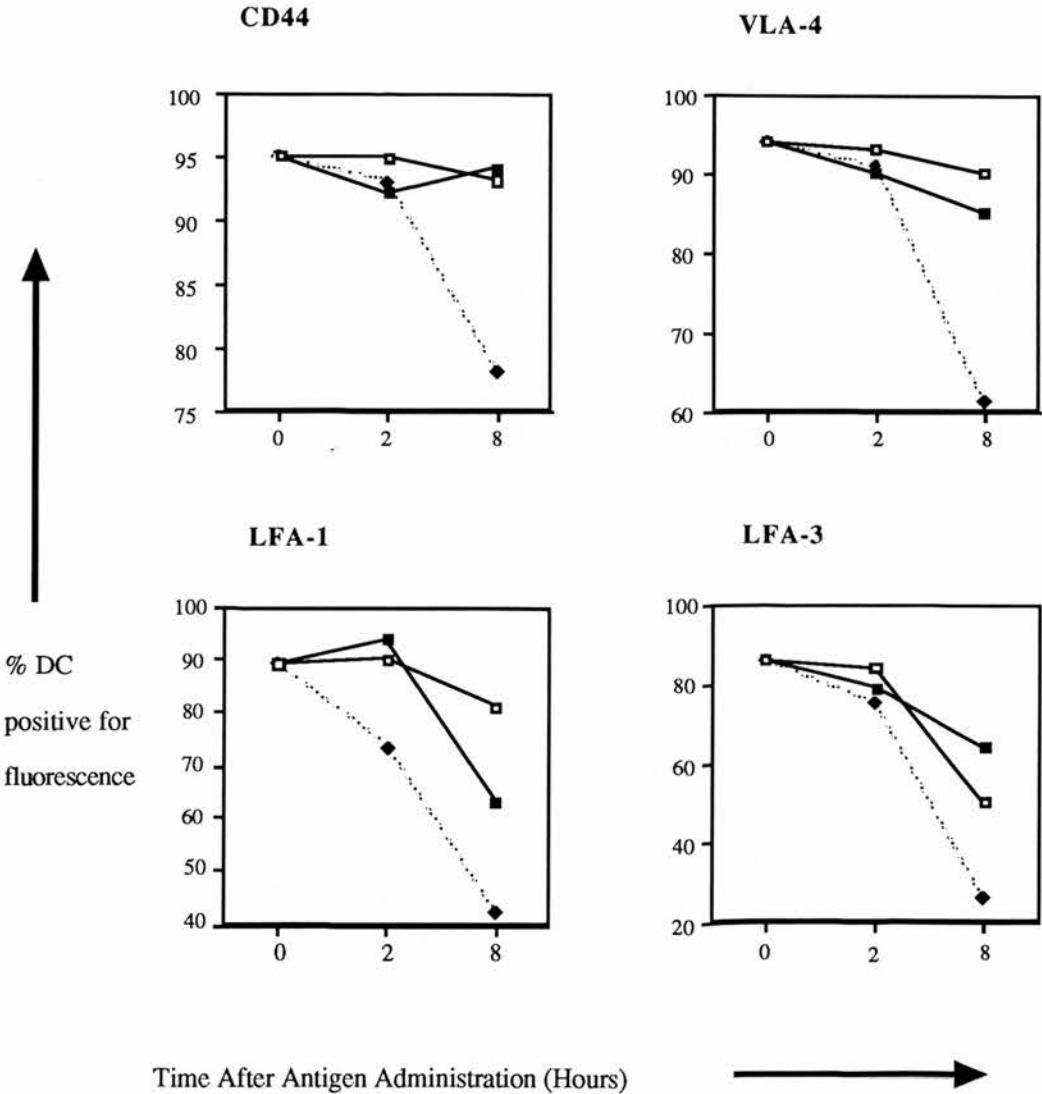
DC in resting afferent lymph constitutively express surface molecules required for antigen presentation and cellular interactions such as adhesion and costimulation. Flow cytometry analysis demonstrated the presence of MHC class I and class II products, necessary for presentation of antigen to CD8 and CD4 T cells respectively (Unanue 1984). CD1, which may be involved in the presentation of antigens to $\gamma\delta$ T cells (Porcelli et al 1989), was also present.

LFA-1 on the DC surface interacts with ICAM-1 and ICAM-2 on the T cell (Springer 1990). DC also express ICAM-1 and ICAM-2 which bind T cell LFA-1. Binding to both LFA-1 and ICAM-1 delivers an activational signal to the T cell; this ligand pair is important in costimulation. LFA-1, which increases in affinity for its ligands after being phosphorylated on activation of the cell (Valmu et al 1991), was detected on DC from sheep afferent lymph, but no monoclonals were available to investigate ICAM-1 and ICAM-2 expression. LFA-1 and its ligands are thought to be involved in the initial binding events in DC clustering and activation of resting T cells (Inaba & Steinman

Figure 20

Afferent lymph cells from a sheep primed to OVA were cultured either in medium, with OVA ($1\mu\text{gml}^{-1}$), or with OVA ($1\mu\text{gml}^{-1}$) and anti-OVA antibodies ($30\mu\text{gml}^{-1}$). DC were analysed for expression of the adhesion molecules CD44, VLA-4, LFA-1 and LFA-3 before culture, and at 2 and 8 hours of culture at 37°C . The monoclonal antibodies were detected with a FITC conjugate; the results are the percentage of DC positive for FITC fluorescence at each time point.

FIGURE 20 DC Expression of Adhesion Molecules After *In Vitro* Culture



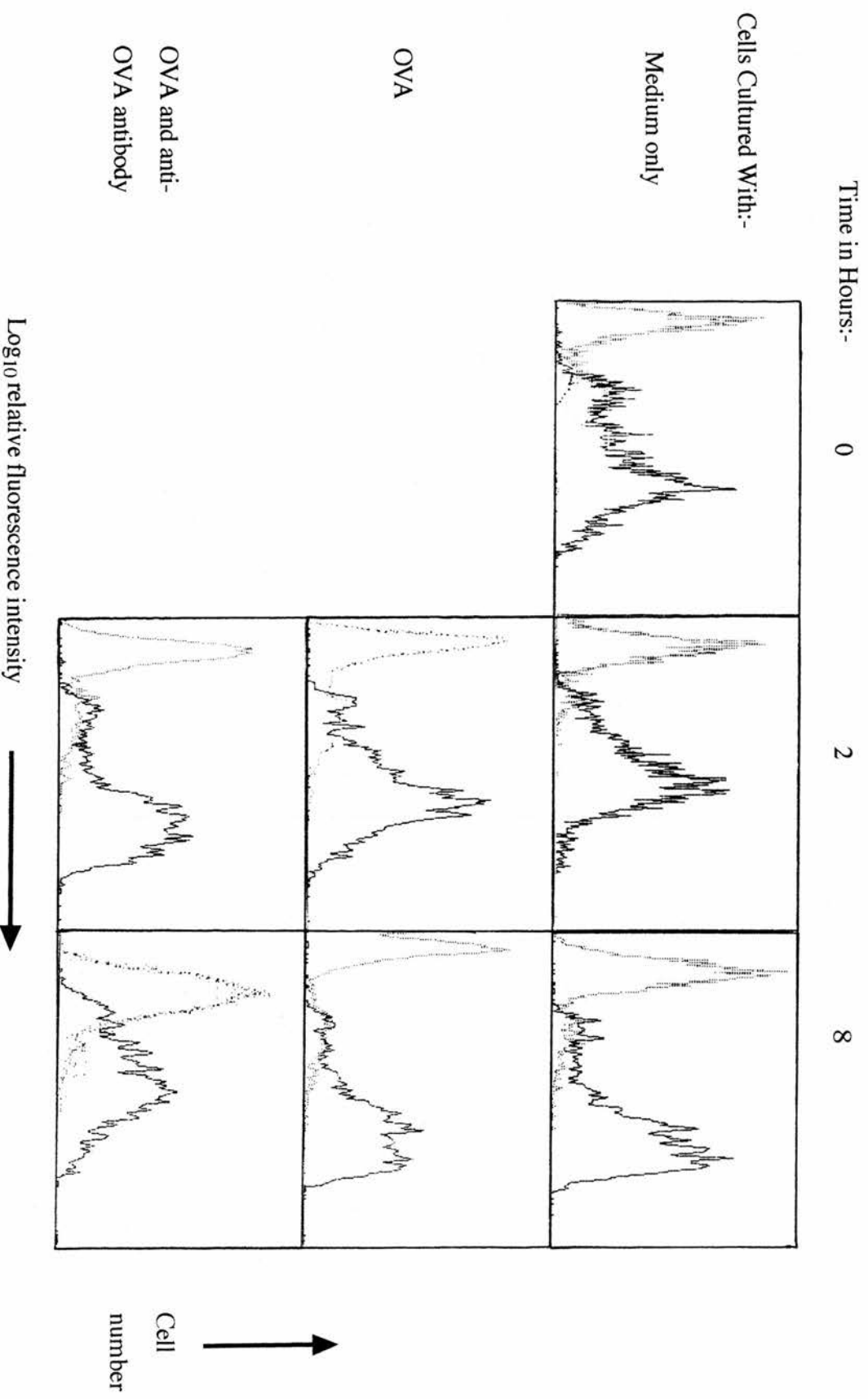
Key

- medium only
- OVA
- ◆— OVA and anti-OVA antibody

Figure 21

Afferent lymph cells were cultured *in vitro* as described, and stained with a CD44-specific MAb at various time points. The negative control profile (dotted lines) illustrates staining with VPM53.

FIGURE 21 Flow Cytometry Profiles of DC Staining for CD44 on *In Viro* Culture



1987).

The Ig superfamily molecule LFA-3 and its ligand CD2 were both expressed by afferent lymph DC. CD2 is usually restricted to T cells, where it is important in cell activation (Dustin & Springer 1989). Costimulatory signals are provided to the T cell on binding of CD2 with LFA-3 or certain anti-CD2 monoclonals. LFA-3 is more widely distributed and is found on leucocytes and endothelial cells, suggesting a role in cell migration. LFA-3 and CD2 are also important in DC-T cell clustering (King & Katz 1989). It is possible that DC acquire CD2 molecules passively by interaction with LFA-3 (Bujdoso et al 1990). Alternatively, CD2 may be produced by DC and could play a role in the activation of these cells. Analysis of mRNA should determine whether CD2 is synthesised by DC.

The $\beta 1$ integrins VLA-4 and VLA-6 interact with ligands in the extracellular matrix and facilitate cell migration and homing (Hemler 1990). VLA-4 binds fibronectin, while VLA-6 binds laminin. VLA-4 also interacts with VCAM-1 on cells such as endothelial cells, tissue macrophages and DC. Binding of ligands to the integrins on a T cell delivers a costimulatory signal (Shimizu et al 1990, Davis et al 1990). VLA-4 was detected on DC from the afferent lymph of sheep by FACS analysis. It may be an important molecule in the migration pathways of DC, and could also provide activational stimuli on ligand binding. VLA-6 is probably also involved in migration through the extracellular matrix, but is expressed only by a subset of DC in afferent lymph.

CD44 is an adhesion molecule with widespread distribution, found on B and T cells, monocytes and neutrophils as well as epithelial cells and fibroblasts. CD44 binds hyaluronate; homotypic adhesion may also occur (Belitsos et al 1990). Binding of ligand to CD44 provides an activational stimulus to the cell, and CD44 expression on sheep lymphocytes is upregulated on culture/ activation *in vitro* (Mackay et al. 1988b). In the experiments described here, CD44 was expressed at consistently high levels by afferent lymph DC.

CD4 was also found on sheep DC. This T cell marker has previously been reported on ovine afferent lymph DC (Bujdoso et al 1990), and has recently been demonstrated on DC from human blood (O'Doherty et al 1993). The role of CD4 on DC may involve its interaction with MHC class II on activated T cells, mediating adhesion and providing stimulatory signals to the DC.

Surface immunoglobulin was found on a variable proportion of DC in the experiments described here, as has previously been reported (Miller & Adams 1977). This was mainly of the IgM isotype, with a small proportion of DC positive for surface IgG1. The significance of SIg on DC in resting afferent lymph is unclear. IgM antibodies may act as a concentrating mechanism for the small amounts of antigen present in the primary immune response, attaching to the DC membrane via μ Fc receptors or complement receptors. However, IgM is not simply adsorbed onto the DC surface when DC bearing low levels of SIgM are incubated in lymph *in vitro* before flow cytometry analysis. The small percentage of DC positive for IgG may reflect a low level of expression of Fc γ RI by these cells (see Chapter 4), which would enable them to bind uncomplexed IgG.

3.7.2. Antigen Uptake

When incubated at room temperature with FITC-OVA a small proportion of DC accumulated antigen (generally <30%). Enhanced uptake of fluorescent antigen by DC in the presence of specific antibody was demonstrated *in vivo* and *in vitro*. Similar results were observed in experiments with alveolar macrophages, which are known to express FcR for IgG. The degree of enhancement varied with the antibody/ antigen ratio in both cases. An increase in DC surface IgG was also seen under conditions that promoted antigen uptake, consistent with the detection of IgG antibody in immune complexes on the DC membrane. No change in IgG levels was seen when these cells were incubated with OVA alone. The percentage of DC positive for surface IgG was less than the percentage which took up FITC-OVA in the presence of specific antibodies; however the experiments were separate, and unlabelled OVA was used in the IgG analysis. The fluorescent OVA was also directly labelled, while IgG on the cell

surface was indirectly labelled by two layers of reagents.

Anti-OVA antibodies of the IgM isotype did not enhance the uptake of antigen by afferent lymph DC *in vitro*. Sheep serum containing these antibodies had no effect on the uptake of FITC-OVA by DC, while in the same experiment lymph containing IgG anti-OVA antibodies caused increased antigen uptake. Affinity purified IgM anti-OVA antibodies were also ineffective. The reason for this is unclear; DC have variable amounts of IgM on their surfaces, which may block further uptake. The method of attachment of IgM to the DC has not been determined; a μ FcR or complement receptor may be involved. If IgM is taken up via complement receptors, a lack of complement in the *in vitro* experiment may explain the failure of these cells to take up IgM-OVA immune complexes. Alternatively, it may be the 7S subunit of IgM rather than the 19S molecule which is involved in antigen interactions with DC; these two molecules may be taken up via different receptors and the monoclonal antibody VPM13 would not distinguish between the two. Another possibility is that IgM antibodies do not play a role in antigen uptake by DC and that DC acquire antigen by pinocytosis in the primary immune response. Although no enhanced uptake of antigen was seen in the experiments described above, in the experiments where OVA was administered into the drainage area of a cannulated, primed sheep, increases in the percentage of DC staining for IgM were observed at various time points in four of the five sheep used. This could represent an antigen-induced mechanism of IgM attachment to the DC surface *in vivo*. Further experiments would be required to ascertain whether an increase in the percentage of DC staining for IgM also occurs after antigen administration in a primary response.

3.7.3. Changes in DC Phenotype After Antigen Administration

Alteration in the expression of several DC surface molecules was observed after antigen challenge in a primed sheep. A marked decrease in the percentage of DC positive for both MHC class II and CD1 occurred within three hours of intradermal antigen injection. The percentage of DC positive for each marker then increased up to the 24 hour time point in three of five sheep. The levels of surface Igs were more variable

between experiments, although an increase in numbers of DC positive for IgM and Ig light chain did occur in four of the five sheep, as discussed above. A transient increase in the percentage of afferent lymph DC positive for IgG occurred in two animals. This probably represents IgG-anti-OVA antibodies in immune complexes attached to FcγR on the DC surface. Not all of the surface molecules investigated showed a similar decrease in expression; CD44 stayed at more or less constant levels on DC in the two sheep used for this experiment.

These phenotypic changes may represent an actual decrease in the levels of surface molecules on the DC present in afferent lymph, by downregulation of genes for molecules such as class II and CD1, or by enzyme cleavage of surface molecules. Alternatively, a different population of cells could be entering the lymph after antigenic stimulation. If a decrease in the levels of markers on the same cell population is responsible, this does not affect all surface molecules uniformly, as shown by the minimal alteration in expression of CD44.

A similar decrease in the expression of surface markers on DC occurred during *in vitro* culture. This effect was seen on culture in medium only for all of the markers examined, although the decrease in expression of CD1 and MHC class II was slight. However, a more pronounced decrease in expression, for some markers at least, occurred on culture with antigen and specific antibody. Thus the proportion of DC positive for CD1 and MHC class II after 8 hours was considerably less where cells were cultured with OVA and antibody as opposed to medium or OVA only. In this experiment, the proportion of DC expressing CD44 also decreased, in contrast to the *in vivo* experiment. Levels of VLA-4, LFA-3 and LFA-1 staining also fell. Staining for IgG remained fairly constant on DC cultured in antigen and antibody, probably due to IgG anti-OVA antibodies on the DC surface in the form of immune complexes. IgG levels were decreased on cells incubated in medium or OVA.

The decrease in staining of DC for various surface molecules on *in vitro* culture with OVA and anti-OVA antibody supports the theory that the same population of DC is altering its phenotype after antigen is administered in a secondary response *in vivo*.

Previous work has demonstrated an increase in MHC class II and CD1 expression on afferent lymph DC 2-5 days after antigen administration in a primed animal (Hopkins et al 1989). The increase in MHC class II expression correlated with more effective accessory functions of the DC. The more immediate changes in expression of MHC class II observed in the experiments described here may be a mechanism to turnover the MHC molecules with peptides present on the DC surface at the time of antigen administration, allowing newly synthesised MHC molecules to interact with and present peptides of the administered antigen (i.e. OVA). A similar mechanism could account for the alterations in CD1 expression.

The explanation for the decrease in expression of most of the adhesion molecules examined is less clear. Downregulation of certain surface molecules may be required to effect the movement of antigen-bearing DC from the dermal tissues to the draining lymphatics. This is discussed further in Chapter 7.

CHAPTER FOUR

EXPRESSION OF FC γ R BY AFFERENT LYMPH DC

4. EXPRESSION OF Fc γ R BY AFFERENT LYMPH DC

4.1. Introduction

The aim of the work described in this chapter was to investigate Fc γ R expression by DC using Western blotting of cell lysates and flow cytometry analysis. As there were no anti-sheep Fc γ R mAbs available, it was decided to raise rabbit antisera against each of two peptide sequences from the extracellular domains of bovine Fc γ RI and an IgG-Sepharose matrix with attached putative ovine Fc γ R. These antisera were then used in Western blot analysis of afferent lymph DC and other ovine cells, and in flow cytometry analysis of DC in resting lymph and following the administration of antigen *in vivo* and *in vitro*.

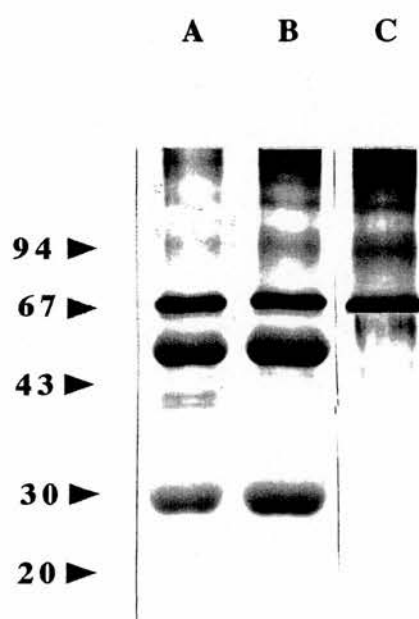
4.1.1. R221 Antiserum

The R221 antiserum was raised against the column material produced in the attempt to purify ovine Fc receptors for IgG from alveolar macrophage lysate. The experimental details are in section 2.7.4. The lysate was passed repeatedly through a BSA-sepharose pre-column followed by the specific IgG-sepharose column. This column was washed and then acid-eluted. No protein was eluted from the IgG-sepharose column, as assessed by SDS-PAGE analysis of the fractions collected (not shown). However, when the column material was boiled in SDS reducing buffer and the supernatant was run on a slab gel under reducing conditions, a protein doublet was visualised at around 40kD which was not seen with the BSA-sepharose column or IgG-sepharose which had BSA only passed through it (Fig.1). This was the correct size for Fc γ RII, which in humans and mice has a molecular weight of 40kD. IgG-sepharose with the attached 40kD protein was emulsified in Titermax adjuvant and used to immunise a rabbit as described in Materials and Methods.

Figure 1

IgG-Sepharose and BSA-Sepharose were made as described in Section 2.8.1. The lysate prepared from alveolar macrophages was passed through these columns, which were eluted at acid pH. The column materials were then boiled in SDS reducing buffer and the supernatants run on SDS-PAGE gels. Track A shows the IgG-Sepharose after recirculation of macrophage lysate, track B represents IgG-Sepharose which has had only BSA passed through it, and in track C BSA-Sepharose from the pre-column was run. The positions of the low molecular weight markers are shown.

FIGURE 1 SDS-PAGE Analysis of IgG-Sepharose After
Passage of Alveolar Macrophage Lysate



A - IgG Sepharose + macrophage lysate

B - IgG-Sepharose

C - BSA-Sepharose

4.1.2. R220 Antiserum

The sequence for bovine Fc γ RI has recently been published (Fig.2; reference Symons & Clarkson 1992), while no sequence data for ovine Fc γ Rs is currently available. There is approximately 60% homology at the amino acid level between the extracellular domains of the murine, human and bovine Fc γ RI: it is therefore likely that a greater degree of homology will exist between the ovine and bovine Fc γ RI, and that antisera raised against peptides from the bovine sequence will cross-react with ovine Fc γ Rs.

Rabbit 220 was immunised with FcR peptide 1 (FP1), a fifteen amino acid peptide from a hydrophilic region of the second extracellular domain of bovine Fc γ RI with the following sequence:-

Cys-Ser-Gly-Glu-Arg-Arg-Arg-Arg-Tyr-Thr-Ser-Ala-Gly-Gly-Ser

R220 antiserum was initially tested against FP1 by ELISA; a titre of 1/1280 was obtained. Anti-FP1 antibodies were affinity purified from the R220 antiserum using an FP1-Sepharose column. The antibodies were tested by ELISA for anti-OVA activity, and had a titre of 1/160.

4.1.3. R225 Antiserum

This serum was raised against FcR peptide 2 (FP2), a thirteen amino acid peptide from the first extracellular domain of bovine Fc γ RI with the following sequence:-

Cys-Glu-Gly-Pro-His-Arg-Pro-Gly-Asp-Thr-Ala-Thr-Gln

The antiserum was tested by ELISA before use, and had an anti-FP2 titre of 1/640.

Figure 2

The amino acid sequence of the extracellular portion of bovine Fc γ RI is shown (Source: Symons & Clarkson 1992), with the regions of FP1 and FP2 outlined in the first and second extracellular domains.

FIGURE 2 Amino Acid Sequence of Bovine FcγRI

	<i>FP2</i>
First Domain	ADPTKAVITLKPPWVSVFQEEENVTL CEGPHRPGDTATQ WFLN GTAIKTLAPRYSINSATFDDSGEYKCCQTGLSMLSDPVQLEIHS
Second Domain	DWLLQVTSRVFTEGDPLALRCHAWKNMPVYKMLFYKDGKP FRSSQDSEFTILQTNLSHNGIYH CSGERRRRYTSAGGS ITIK <i>FP1</i>
Third Domain	ELFPAPVLRTSFSSSPHQEGNLVNLSCETKLPSEKPGQQLYFSF YVGNKTLISRTTSSEYQTFIAKKEDRRLYWCEATTGDGNLIK SPELELPVL

4.2. Western Blots with R220 Antiserum

4.2.1. DC

Lysates of afferent lymph DC were prepared as described in section 2.7.6., from cell populations that were > 92% DC by morphology on Leishmans-stained cytopins. The lysates were fractionated by SDS-PAGE using 5-20% gradient gels, and 10⁵ DC equivalents were blotted with R220 FP1 antiserum and the preinoculation control at 1/100 dilutions in blocking buffer (Fig.3), as described in section 2.7.5.. Specific bands were visible at 72 and 40kD, the latter band being more intense than the former. There were also two fainter bands immediately above the 40kD band on the blot. The two major bands were consistent with FcγRII (40kD) and FcγRI (72kD) as described in other species (Van de Winkel & Anderson 1991). No bands were present on the preinoculation serum control blot (Fig.3).

4.2.2. Alveolar Macrophages

These cells are known to express FcγRI and FcγRII in mice and humans (Unkeless et al 1988), and the blot of the ovine alveolar macrophage lysate supports this, with an intense band visible at 72kD and a less intense band at 40kD (Fig.3). In contrast to the result obtained with the DC lysate, the alveolar macrophages in this sample seemed to express more FcγRI than DC, but a comparable amount of FcγRII. As with the DC blot, there were two fainter bands visible above the 40kD band. Some non-specific bands were visible on the control blot with the preinoculation serum; these were of intermediate size between the 40 and 72kD bands.

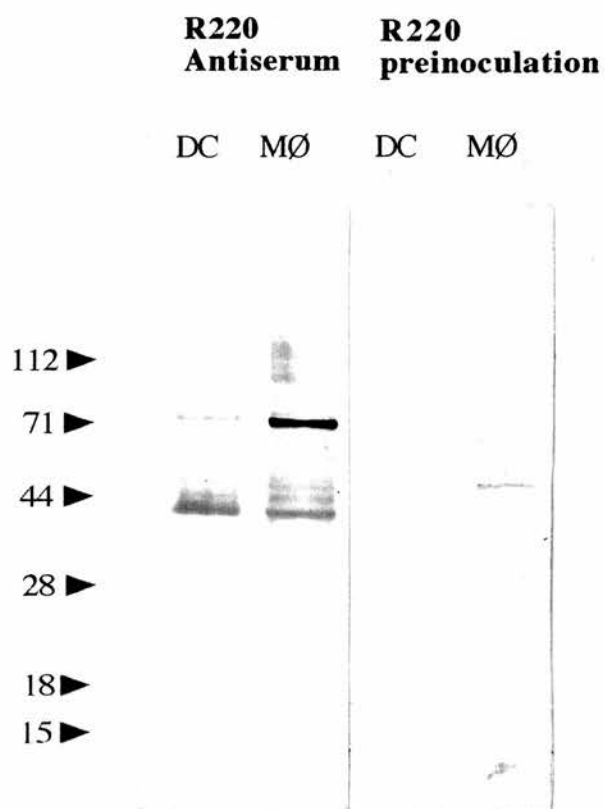
4.2.3. Lymphocytes

Although the DC populations from which lysates were prepared were > 92% DC by morphology, a small percentage of cells (< 8%) were lymphocytes. The contribution of these contaminating cells to the blot of the DC lysate in Fig.3 was assessed by blotting

Figure 3

DC and macrophage lysates were prepared as described. 10^5 cell equivalents in each case were fractionated by SDS-PAGE and analysed by Western blotting. R220 antiserum and the control preinoculation serum were used at a 1/100 dilution. Antibodies were detected using an alkaline phosphatase conjugate and blots were developed with NBT/ BCIP. Prestained low molecular weight markers were used in these blots.

FIGURE 3 Western Blot Analysis of DC and Macrophages with R220 FP1 Antiserum



10⁴ lymphocyte equivalents obtained from efferent lymph, i.e. the number of lymphocytes that would be present if the DC population contained 10% lymphocytes. There were no bands observed when this number of lymphocytes was blotted with the R220 antiserum at a 1/100 dilution (Fig.4., track B). It is therefore unlikely that the number of contaminating lymphocytes in the DC preparations contributed to the two bands observed in Fig.3. Lymphocytes did react with this serum when they were used in greater quantities: 10⁵ efferent lymphocyte equivalents blotted with R220 antiserum gave a faint but visible band at 40kD, indicating the presence of FcγRII on these cells (Fig.4, track A). The source of the lymphocytes used above may be important; this is discussed in section 4.7.

Further blots were carried out on the lysates of lymphocytes from a second source. CD4⁺ T cells were separated from PBMs by positive selection on a MACS, as described in Chapter 2. After lysis, 10⁵ cell equivalents were run in track C of Fig.4, and 10⁴ cell equivalents in track D. A broad smear was visualised between 55-70kD in track C, with a more discrete band in track D. This band may represent FcγRIII, reported to be present on lymphocyte subsets in other species (Daeron et al 1988), with the diffuse appearance due to the extensive glycosylation of the receptor. The 72 and 40kD bands observed on blots of DC and macrophage lysates were not present in the lysates of the CD4⁺ T cells.

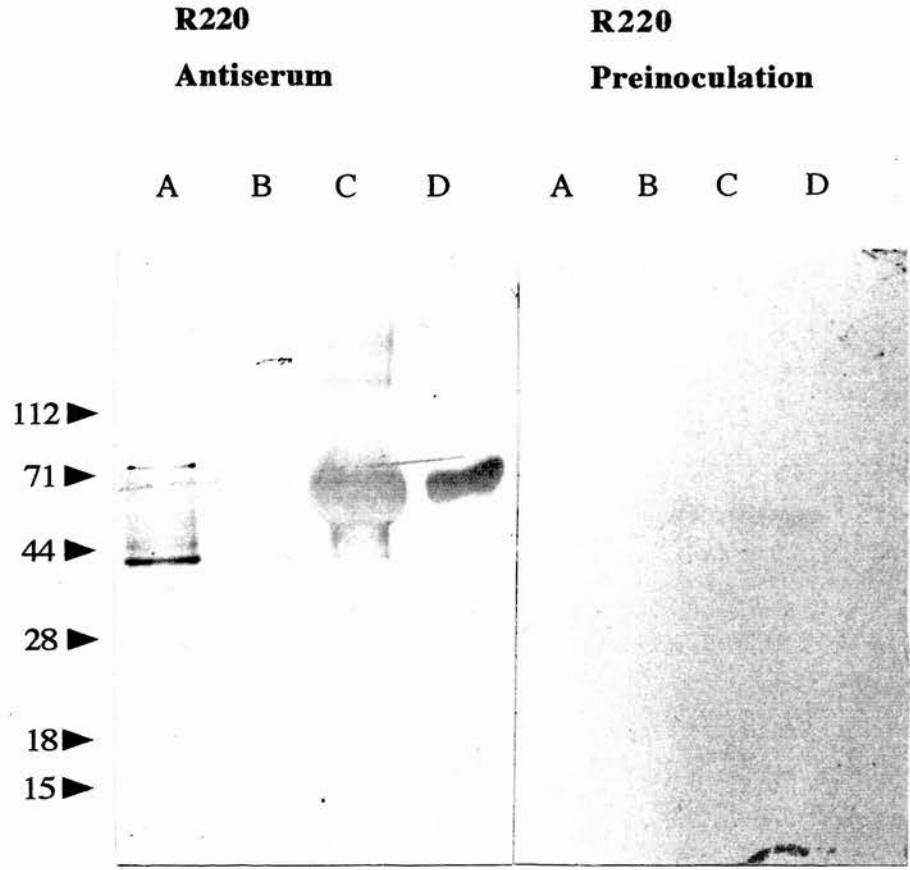
4.3. Western Blot Analysis of Ovine Cells with Affinity Purified Anti-FP1 Antibodies

Anti-FP1 antibodies were affinity purified on an FP-1-Sepharose column and dialysed into PBS before use in blots. The control antibody for these blots was anti-OVA antibody affinity purified from the serum of a rabbit primed to an irrelevant peptide conjugated to OVA, and used at the same Ig concentration. Western blots with the affinity purified antibodies were carried out on lysates of DC, alveolar macrophages, efferent lymphocytes and CD4⁺ T cells (Fig.5). In track A of the blot with anti-FP1 antibodies, where 10⁵ DC equivalents were run, bands were visible at around 40kD

Figure 4

Lysates were prepared from efferent lymphocytes and CD4+ T cells separated from PBMs. Varying numbers of cell equivalents were run on an SDS-PAGE gel for subsequent Western blot analysis to assess the contribution of lymphocytes in DC preparations to the results shown in Fig.3. The positions of prestained low molecular weight markers are shown.

FIGURE 4 Western Blot Analysis of Lymphocytes with R220 FP1 Antiserum



A - 10^5 efferent lymphocytes

B - 10^4 efferent lymphocytes

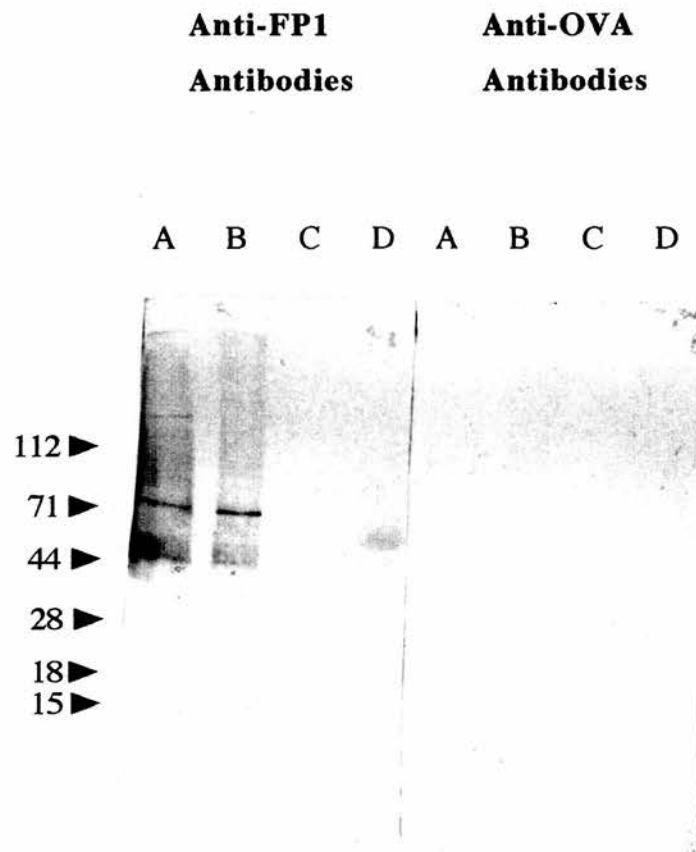
C - 10^5 CD4⁺ T cells

D - 10^4 CD4⁺ T cells

Figure 5

Cell lysates of alveolar macrophages (10^5 cell equivalents), DC (10^5 cell equivalents), efferent lymphocytes (10^4 cell equivalents) and CD4+ T cells (10^4 cell equivalents) were analysed by Western blotting with affinity purified anti-FP1 antibodies. The controls for these blots were the same lysates blotted with affinity purified rabbit anti-OVA antibodies. The positions of prestained low molecular weight markers are shown.

FIGURE 5 Western Blot Analysis of Ovine Cells
With Anti-FP1 Antibodies



A - 10^5 DC

B - 10^5 Macrophages

C - 10^4 efferent lymphocytes

D - 10^4 CD4+ T cells

and at 72 kD. The 72kD band is discrete, while the band at 40kD is diffuse and there may in fact be more than one band present. 10^5 alveolar macrophage equivalents gave the same pattern of bands (track B). These bands were the same sizes as those detected with the R220 antiserum in Fig.3 and were specific, with no bands present on the control blot. There were no bands in track C, in which the lysate of 10^4 efferent lymphocytes was run to represent the contribution of the contaminating lymphocytes in the DC preparation. In track C, 10^4 CD4+ T cells obtained from PBMs were blotted with the affinity purified anti-FP1 antibodies. A faint smeared band of approximate size 50-70kD was present, which could be Fc γ RIII as in Fig.4. The 72kD and 40kD bands seen on the DC and macrophage lysates, which may represent Fc γ RI and Fc γ RII, were not present in the CD4+ T cell lysate blot.

4.4. Western Blot Analysis of the Putative Ovine Fc γ R

The reactivity of R221 antiserum was investigated by Western blot analysis of the IgG-Sepharose with attached putative Fc γ R. The supernatant obtained after boiling the column material with the attached 40kD protein in SDS reducing buffer was fractionated by SDS-PAGE and blotted with the R221 antiserum and preinoculation control at 1/100 dilutions. The antiserum recognised bands of the correct sizes for the IgG heavy and light chains and the 40kD doublet, while the preinoculation serum did not react with any of these bands (Fig.6).

The R220 antiserum was also tested against the same antigenic material. Unexpectedly, the R220 antiserum reacted with the IgG heavy and light chain bands as well as the 40kD doublet (track A, Fig.7). However, the preinoculation serum also reacted with the IgG heavy and light chains, although to a lesser extent, therefore this may be a non-specific effect.

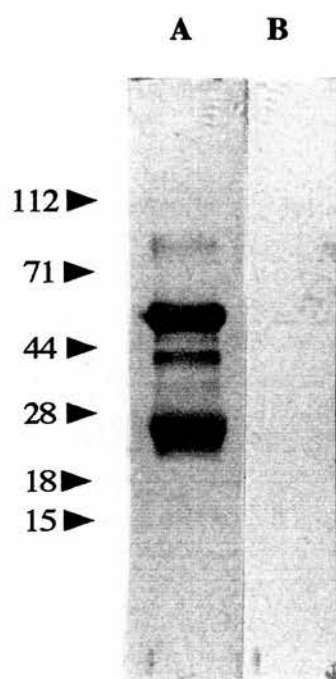
4.5. Cell Lysate Analysis by Western Blotting With R221 Serum

A series of blots was carried out on cell lysates using the R221 antiserum and

Figure 6

Alveolar macrophage lysate was passed through an affinity column in an attempt to purify Fc γ R. 50 μ l of IgG-Sepharose from the column was then boiled in SDS reducing buffer and centrifuged to pellet the insoluble material. The supernatant was fractionated by SDS-PAGE using gradient slab gels for subsequent analysis by Western blot. In this blot, R221 antiserum was used at a 1/100 dilution, with the preinoculation serum at the same dilution as the control.

FIGURE 6 Western Blot of The IgG-Sepharose Column Antigen
With R221 Antiserum



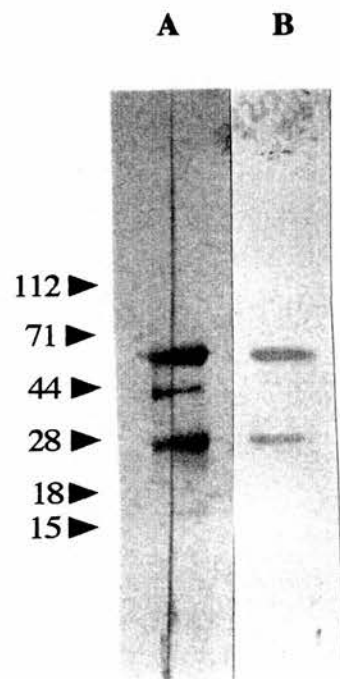
A - Antiserum

B - Preinoculation serum

Figure 7

Alveolar macrophage lysate was passed through an IgG-Sepharose affinity column. 50µl of IgG-Sepharose was boiled in SDS reducing buffer and run on an SDS-PAGE slab gel for subsequent analysis by Western blotting. In this blot R220 antiserum and the preinoculation control serum were used at a 1/100 dilution.

FIGURE 7 Western Blot of The IgG-Sepharose Column Antigen
With R220 FP1 Antiserum



A - Antiserum

B - Preinoculation serum

preinoculation serum. Blotting of the DC lysate with R221 antiserum at a 1/100 dilution gave a major band of molecular weight 40kD and a second at 23kD (Fig.8, track B). No bands were present on the preinoculation serum control blot (track A). Bands of the same weights were visualised on blots of alveolar macrophage lysates (Fig.9). The 40kD band may represent Fc γ RII, as for the blots of the same cell lysates with the R220 antiserum (Fig.3) and the affinity purified anti-FP1 antibodies (Fig. 5). The efferent lymphocyte lysate did not react with this antiserum (Fig.9, track B), but a smeared band is present in the CD4+ T cell lysate lane (track C). This band is of molecular weight 80-90kD, and therefore is not the expected size for any Fc γ R.

4.6. Flow Cytometry Analysis of DC With Rabbit Antisera

The Western blot experiments described above clearly indicate the presence of Fc γ Rs on ovine afferent lymph DC. Flow cytometry analysis was carried out to further investigate DC expression of Fc γ Rs in resting lymph and after antigen administration *in vivo*. Other cell types such as lymphocytes from afferent and efferent lymph and blood, and neutrophils were also analysed for Fc γ R expression.

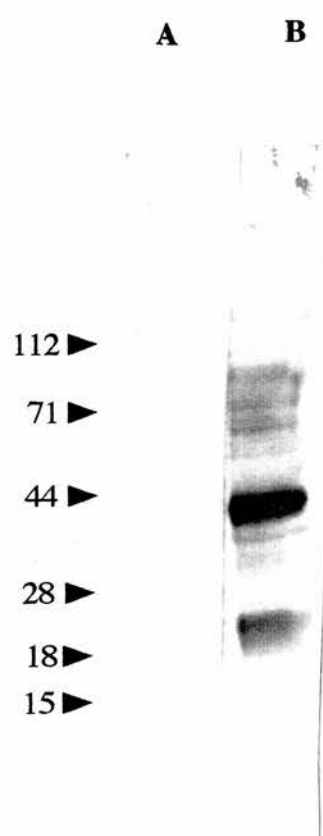
4.6.1. Analysis of ovine cells with R220 FP1 Antiserum

Strong staining of DC was observed with R220 antiserum, as shown in Fig.10, where the antiserum was used at a 1/10 dilution. 78% of DC were positive for FITC fluorescence in this FACS profile; some showed very high levels of fluorescence. The preinoculation serum at the same dilution was used as a negative control. A "tail" of increasing fluorescence was present in the DC stained with the preinoculation serum. To overcome this and to obtain a more discrete negative control peak, affinity purified peptide-specific antibodies were used. After a ten-fold concentration in an Amicon ultrafiltration apparatus to 18 μ gml⁻¹, anti-peptide antibodies were used in immunofluorescence analysis of DC. Affinity purified anti-OVA antibodies were used as a control at the same Ig concentration; the anti-OVA titre of this antibody preparation was determined by ELISA as 1/1280.

Figure 8

A lysate was prepared from DC as described. 10^5 cell equivalents were run on an SDS-PAGE gel in each track; track A was then blotted with the preinoculation control serum at a 1/100 dilution, and track B with the R221 antiserum at the same dilution. The positions of the prestatined low molecular weight markers are shown.

FIGURE 8 Western Blot Analysis of DC Lysate With R221 Antiserum



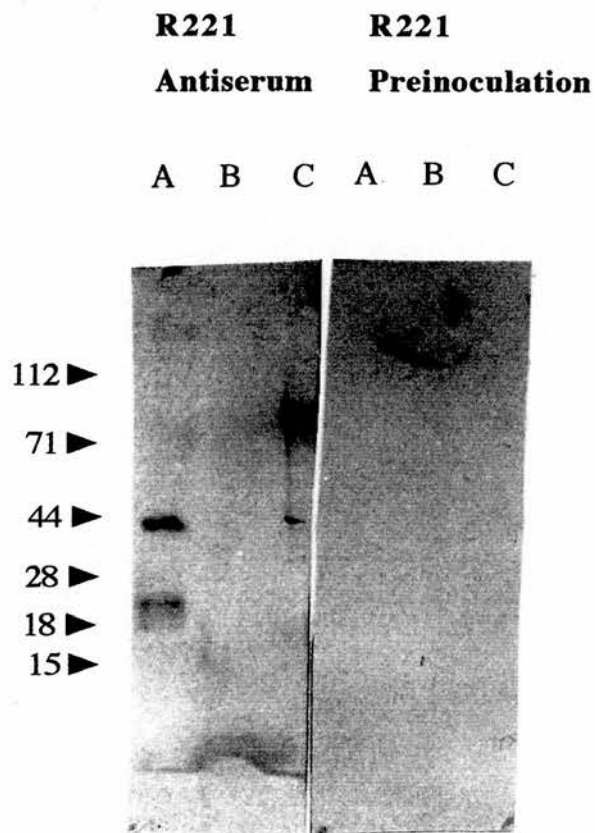
A- Preinoculation serum

B- Antiserum

Figure 9

The cell lysates were prepared as described in Materials and Methods. 10^5 alveolar macrophage equivalents, 10^4 efferent lymphocytes and 10^4 CD4+ T cells were each run in two tracks of an SDS-PAGE gel. The lysates were then blotted with either the R221 preinoculation control serum at a 1/100 dilution, or the R221 antiserum at the same dilution. The positions of the prestained low molecular weight markers are shown.

FIGURE 9 Western Blot Analysis of Ovine Cells With R221 Antiserum



A - Macrophages

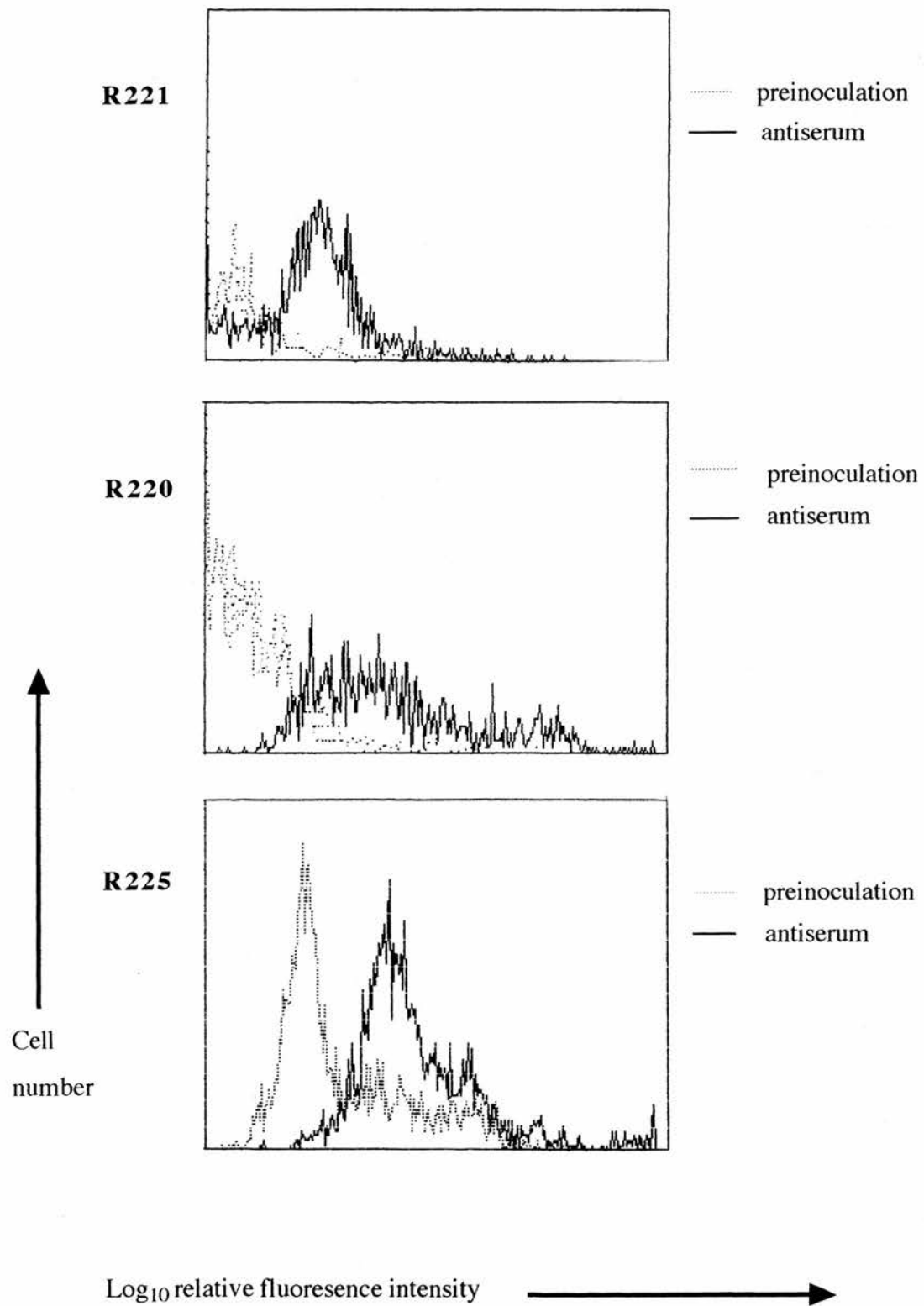
B - Efferent Lymphocytes

C - CD4+ T Cells

Figure 10

Afferent lymph cells were stained for flow cytometry using rabbit antisera and the relevant prebinoculation sera as controls. Antisera and controls were diluted 1/10 in PBA before use. Rabbit 221 (R221) was immunised with the IgG-Sepharose plus putative ovine Fc γ R, R220 with FcR peptide 1 and R225 with FcR peptide 2. Overlay histograms show the staining observed with both prebinoculation sera and antisera.

FIGURE 10 DC Staining With Rabbit Antisera



As illustrated in Fig.11, the affinity purified anti-FP1 antibodies stained most afferent lymph DC while the control antibodies did not. Setting a marker to the right of the negative control peak, the mean percentage of DC staining with the anti-peptide antibodies staining was 56%, with a range of 38% to 76% DC positive for FL-1. However, there was a large degree of overlap between the positive and negative peaks, and it was therefore impossible to assess accurately the percentage of DC positive. In Fig.11, the whole peak representing DC staining with anti-FP1 antibodies has shifted to the right compared with the negative control peak, indicating that all of the cells are expressing FcγR at a low intensity.

Unfractionated R220 antiserum also stained lymphocytes from afferent and efferent lymph and blood, and neutrophils (Fig.12). It was therefore surprising that very little staining of afferent lymphocytes was observed with the affinity-purified anti-FP1 antibodies (Fig.11).

4.6.2. Flow Cytometry Analysis with R221 Antiserum

The antiserum from rabbit 221 stained a large proportion of afferent lymph DC, afferent lymphocytes, efferent lymphocytes and neutrophils. The background level of fluorescence was determined by staining with the preinoculation control serum at the same dilution (Fig.10 and Fig.12). 85% of afferent lymph DC were positive for R221 serum staining in the FACS profile shown in Fig.10, with both antiserum and prebleed used at a 1/10 dilution. Some afferent lymph DC showed very high levels of FITC fluorescence after staining with the R221 antiserum at this dilution (Fig.10)..

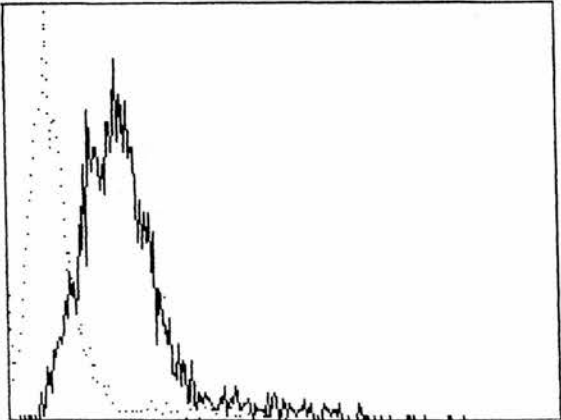
The immunogen administered to the rabbit contained sheep IgG as well as the putative FcγR, thus a proportion of the antibody present in the R221 antiserum was likely to be directed against IgG. This could have contributed to the staining observed on DC, although in repeated flow cytometry analysis of these cells the mean percentage of DC positive for IgG was only 9, with a range of 2-23% (Table 1, Chapter 3). The R221 antiserum may also have contained antibodies directed against sheep Ig light chain, which could potentially account for more of the staining observed on DC; a mean of

Figure 11

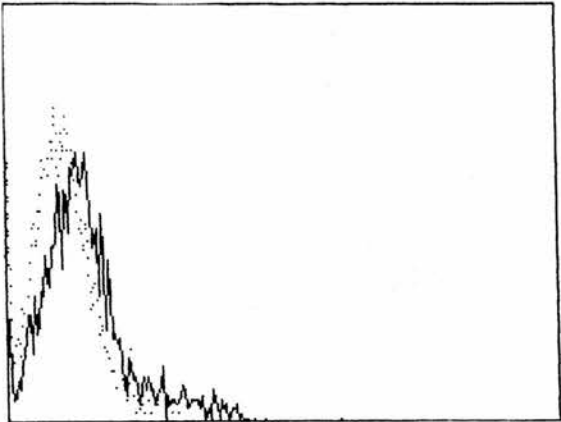
Afferent lymph cells were washed and stained with either affinity purified anti-FcR peptide 1 antibodies or affinity purified anti-OVA antibodies at the same concentration ($18\mu\text{gml}^{-1}$) as a control. The cells were then analysed by flow cytometry, with the DC and lymphocyte populations gated on using the scatter profiles of afferent lymph cells. Ten thousand cells were analysed per sample.

FIGURE 11 DC and Lymphocyte Staining With Affinity
Purified Anti-FcR Peptide 1 Antibodies

DC



**Afferent
lymphocytes**



↑
Cell
number

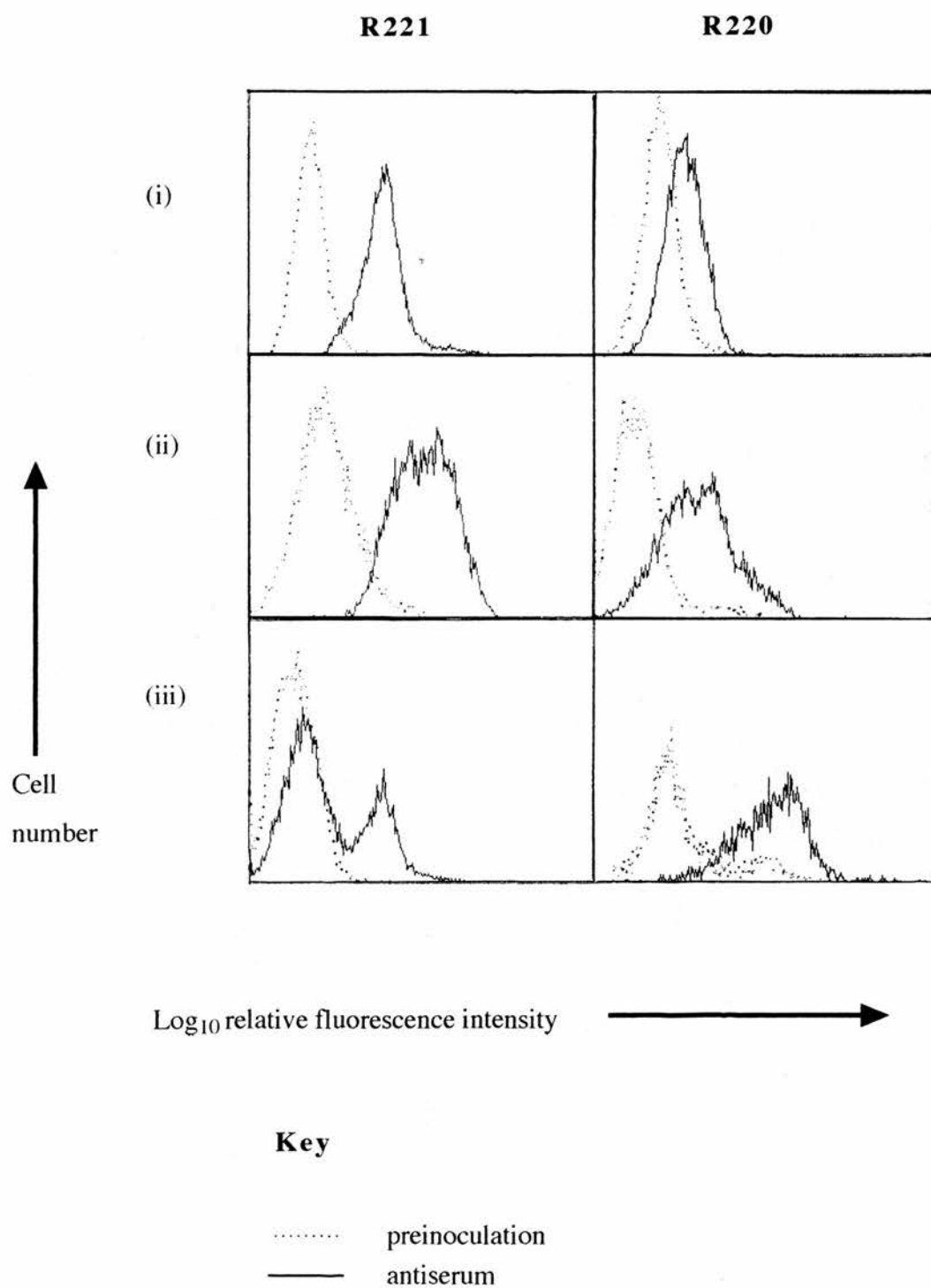
Log₁₀ relative fluorescence intensity →

- Control antibody
- Anti-FcR peptide 1 antibody

Figure 12

Ovine cells were prepared for analysis by flow cytometry, using the preinoculation sera and antisera of rabbits 221 and 220. WBCs were obtained from whole blood by ammonium chloride lysis of erythrocytes as described in Chapter 2, then neutrophils (i) and lymphocytes (ii) were gated on according to their scatter profiles. In (iii), staining of efferent lymphocytes by R221 preinoculation serum and antiserum and afferent lymphocytes by R220 preinoculation serum and antiserum are shown. Ten thousand cells were analysed per sample.

FIGURE 12 Ovine Cells Stained With Rabbit Antisera



51% of DC expressed Ig light chain with a range of 12-85% (Table 1, Chapter 3). The absorption of anti-IgG activity from R221 antiserum was attempted by its repeated passage through an IgG-Sepharose column. The IgG-absorbed serum showed only trace staining on afferent lymph DC (not shown). Western blot analysis of the IgG-sepharose column material and putative Fc γ R with the unabsorbed R221 serum showed activity against IgG heavy and light chains and the 40kD protein (Section 4.4), while blots of the same material with the IgG-absorbed R221 antiserum demonstrated residual anti-IgG activity, and reduced reactivity with the 40kD putative Fc γ R (not shown). The absorption procedure therefore seemed to have non-specifically removed antibodies directed against the putative Fc γ R, while failing to remove anti-IgG activity. A Western blot of DC lysate with the unabsorbed R221 antiserum showed strong reactivity with a 40kD protein, but little or no reactivity with IgG heavy chain or Ig light chain (Section 4.5). It is therefore highly probable that the staining of DC demonstrated by FACS analysis and shown in Fig.10, is attributable to the presence of the 40kD putative Fc γ R on the DC surface.

4.6.3. Flow Cytometry Analysis of DC with R225 Antiserum

Initial FACS analysis of DC showed a high background fluorescence with the preinoculation serum. IgG was separated from both preinoculation and antiserum samples by ion-exchange chromatography on a DE52 column in an attempt to decrease non-specific staining. IgG concentrations were determined from OD readings at 280nm and preinoculation and antiserum IgG were used at a concentration of 10 μ gml⁻¹ for further FACS analysis. Positive staining of a large proportion of afferent lymph DC was seen using the R225 antiserum IgG preparation (Fig.10).

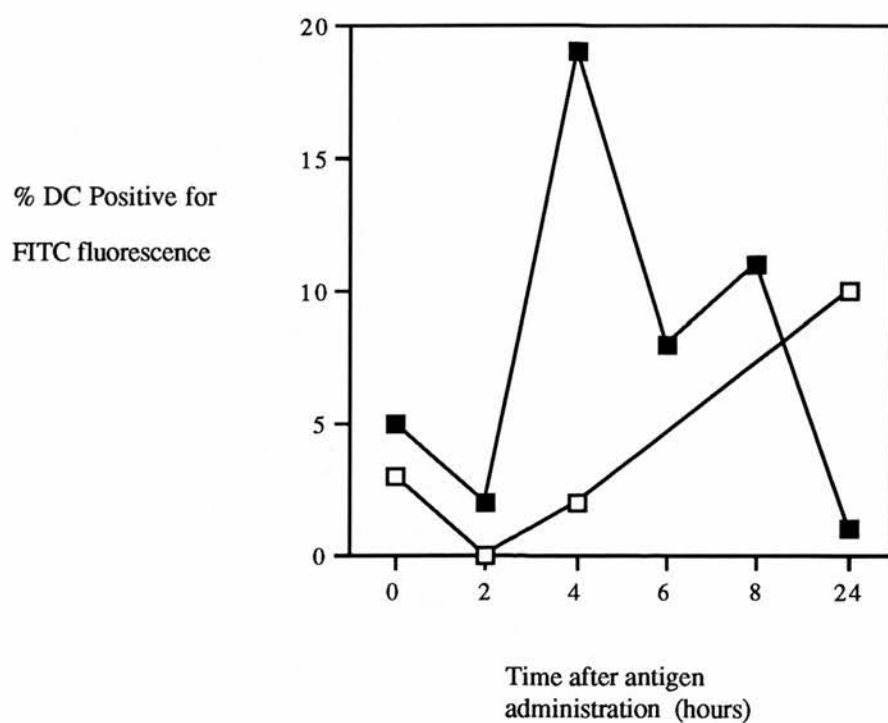
4.6.4. DC Staining with R220 Antiserum Following Antigen Administration in vivo

The R220 FP1 antiserum was included in two phenotyping experiments (Fig. 13). Each was used at a dilution of 1/100 in BSA, rather than at the 1/10 dilution used

Figure 13

DC were collected at various time points after antigen injection into the drainage area of two cannulated OVA-primed sheep (numbers 150 and 151), and stained with rabbit antiserum 220 followed by a FITC-conjugate. Unfractionated afferent cells were stained; DC were gated on as described in Chapter 2. The control was preinoculation rabbit serum at the same dilution as the antiserum. The results are the percentage of DC positive for FITC fluorescence above control staining.

FIGURE 13 DC Staining With Rabbit Antiserum to FcγR Peptide 1
After *In Vivo* Antigen Administration



Key

- Sheep 151
- Sheep 150

previously, in an attempt to obtain lower background staining with the prebleed control, and lose the “tail” of increasing fluorescence observed on staining DC with the 1/10 dilution of the prebleed (Fig.10). Serum was not absorbed against OVA. R220 antiserum showed very little initial staining on DC in both experiments, probably because of the greater dilution of antiserum used. However, the percentage of DC staining positive with the antiserum increased four-fold at the 4 hour time point in sheep 151, and two-fold by 24 hours after antigen administration in sheep 150. Increases in the percentage of DC staining with R220 antiserum may reflect an upregulation of Fc γ R on the DC surface after antigen injection; however, as the antiserum was not absorbed against OVA, rabbit anti-OVA antibodies could be binding to OVA exposed on the DC membrane and contributing to the increase detected. To determine the contribution of the rabbit anti-OVA antibodies, the experiment could be repeated using rabbit antiserum from which anti-OVA antibody activity had been removed.

4.7. Discussion

4.7.1. Western Blot Analysis With Rabbit Antisera

The R220 antiserum was raised against the peptide FP1, the sequence of which was from the second extracellular domain of bovine Fc γ RI. This antiserum reacted with two bands of molecular weight 40 and 72kD on the lysate of alveolar macrophages, a cell type known to express Fc γ RI and Fc γ RII in mice and humans (Ravetch & Kinet 1991). The bands observed were of the correct sizes for Fc γ RII (40kD) and Fc γ RI (72kD). Ovine afferent lymph DC also expressed these molecules, as demonstrated by Western blot analysis of DC lysates. DC appeared to express a greater quantity of Fc γ RII than Fc γ RI. The affinity purified anti-FP1 antibodies reacted with antigens of the same sizes as the R220 antiserum, on both DC and macrophage lysates, while the control antibodies did not pick up these bands.

Afferent lymph cells were subjected to two separations over metrizamide to obtain a DC population of sufficient purity for Western blot analysis. However, a proportion of lymphocytes remained in the DC preparation used for the production of lysates. The

DC population contained <8% lymphocytes, determined by cell morphology on cytopins. Lymphocytes in other species express Fc γ R: Fc γ RII is found on B cells, while Fc γ RII and Fc γ RIII have been reported on subsets of T cells (Lanzavecchia 1990, Daeron et al 1988, Mantzioris et al 1993). To assess the contribution of the residual lymphocytes in DC preparations to these blots, the number of lymphocyte cell equivalents that would be present if the DC population contained 10% lymphocytes was blotted with R220 antiserum and the affinity purified anti-FP1 antibodies. Efferent lymphocytes were used because of the difficulty in obtaining a population of afferent lymphocytes free of DC. There were no bands present on Western blots of efferent lymphocytes with the R220 antiserum or the anti-FP1 antibodies, and the contaminating lymphocytes were therefore unlikely to affect the results of blots on DC lysates. However, when the number of lymphocyte equivalents was increased 10-fold, a band was visualised at 40kD, indicating the presence of Fc γ RII on these cells.

The source of the lymphocytes used in these control blots may be important. Efferent lymphocytes were used in place of the afferent lymphocytes which would actually be present in the DC samples. Afferent lymph T cells are memory cells and are activated, while naive, unactivated T cells are found in resting efferent lymph (Mackay et al 1990). Although subsets of activated T cells in other species express Fc γ R, the strength of the signal detected in the DC lysate and the small numbers of T cells present make a significant contribution by contaminating cells to the bands observed highly unlikely.

Lymphocytes from another source were also examined. Ovine afferent lymphocytes are mainly T cells (Mackay et al 1992), so CD4+ T cells were obtained from PBMs by MACS separation. Western blot analysis of these cells with R220 antiserum and anti-FP1 antibodies demonstrated the presence of a diffuse band of molecular weight 55-70kD. This may be Fc γ RIII, reported on T cell subsets in humans (Sandor & Lynch 1993), with the smeared appearance of the band due to the extensive glycosylation of the receptor (Van de Winkel & Capel 1993). The bands seen at 40 and 72 kD in blots of DC lysates were not present; these CD4+ T cells therefore did not express Fc γ RII at

detectable levels. The expression of Fc γ RIII by CD4+ T cells in humans has been associated with activation of these cells (Sandor & Lynch 1993). The positive selection of CD4+ T cells from PBMs, involving the use of an anti-CD4 monoclonal antibody, may provide an activational signal to the selected cells, causing an upregulation of certain surface molecules. Further experiments, including flow cytometry of CD4+ T cells with R220 and R221 antisera, and Western blot analysis of negatively selected CD4+ T cells would be required to ascertain whether these cells constitutively express Fc γ RIII.

The R221 antiserum, raised against IgG-Sepharose with the attached 40kD protein, blotted with bands of molecular weight 40kD and 23kD on DC lysates. The 40kD band is likely to be Fc γ RII, as discussed above for the blots with R220 antiserum and the affinity purified anti-FP1 antibodies. The identity of the 23kD band is unclear. Soluble forms of Fc γ R have been reported, which lack transmembrane and cytoplasmic portions (Sautes et al 1991). These are of lower molecular weight than the 40kD Fc γ RII, and are present in serum (Fridman et al 1992) and probably also lymph . The 23kD band could therefore represent a soluble form of Fc γ R present in the DC lysate. However, if this is the case it is surprising that the R220 antiserum did not react with this band on the same cell lysates. Western blot analysis of ovine serum and lymph proteins with the R220 and R221 antisera would be informative. The two bands were also present on blots of alveolar macrophage lysate, but were not observed on blots of efferent lymphocytes or CD4+ T cells. A 40kD band was seen on blots of the efferent lymphocyte lysate with R220 antiserum, as described above; however in the blot with the R221 antiserum fewer cell equivalents were used.

Western blot analysis of the immunogen administered to rabbit 221 with R221 antiserum revealed bands corresponding to the heavy and light chains of IgG, and the 40kD protein attached to the IgG-Sepharose matrix. Blotting of the same material with R220 serum revealed the same pattern of bands, while the R220 preinoculation control serum also reacted with the IgG bands but not the 40kD band. This suggests that the R220 antiserum specifically recognises the 40kD putative ovine Fc γ RII.

The above data therefore provide evidence for the expression of Fc γ Rs by afferent lymph DC. Fc γ RII, which binds complexed IgG efficiently but does not detectably bind monomeric IgG, would be responsible for the enhanced uptake of antigen in complex form described in Chapter 3, and the functional consequences in terms of antigen presentation by DC (Chapter 6). The presence of Fc γ RI on the DC surface could account for the low levels of IgG detected on DC from resting lymph by flow cytometry analysis.

The R220 antiserum was raised against a peptide sequence from the second extracellular domain of bovine Fc γ RI. This antiserum was expected to cross-react with ovine Fc γ RI, as Fc γ RI is conserved between species, with the identity of the amino acids in the three extracellular domains between the bovine, murine and human receptors being 60% for the first domain, 58% for the second and 58% for the third domain (Symons & Clarkson 1992). However, this antiserum also reacted with a band of the correct size for Fc γ RII on macrophages, DC and lymphocytes. This indicates that the antiserum cross-reacts with Fc γ RII, and it may also cross-react with Fc γ RIII on CD4+ T cells. While Fc γ RI has three extracellular domains, Fc γ RII and Fc γ RIII have only the first two (Unkeless et al 1988). The first two extracellular domains of Fc γ RI show 40% identity with the extracellular domains of Fc γ RII and Fc γ RIII in mice (Hulett et al 1991) and 50 % in humans (Allen & Seed 1989). The corresponding region of the FP1 sequence in mice shows 40% identity, 60% similarity between Fc γ RI and Fc γ RII, and 33% identity and 60% similarity between Fc γ RI and Fc γ RIII (Fig.14). These regions of murine Fc γ RII and Fc γ RIII have 87.5% identity and similarity. In humans the identity between the different Fc γ R types in this region is considerably less, with 27% identity and 47% similarity between Fc γ RI and Fc γ RII and 27% identity and 40% similarity between Fc γ RI and Fc γ RIII (Fig.14). The corresponding data for the bovine Fc γ Rs is unknown, but would probably relate more closely to the situation in sheep. If the ovine Fc γ R types have a percentage identity in the FP1 region similar that of the murine Fc γ Rs, then this could account for the cross-reactivity of the antiserum raised against the bovine Fc γ RI peptide.

Figure 14

The amino acid sequences of human and murine Fc γ Rs are compared in the region of FP1. The sequences were obtained from the Swissprot database, and the percentages for identity and similarity were calculated using the programme "Gap".

FIGURE 14 Sequence Comparisons of Human and Murine FcγRs in the
Region Corresponding to the FcR peptide 1 Sequence

(i) Murine	% identity	% similarity
<u>FcγRI vs FcγRII</u>		
CSGT. GRHRYTSAGVS : : CKGSLGRTLHQSKPVT	40	60
<u>FcγRI vs FcγRIII</u>		
CSGT. GRHRYTSAGVS : : CKGSLGSTQHQSHPVT	33.3	60
<u>FcγRII vs FcγRIII</u>		
CKGSLGRTLHQSKPVT CKGSLGSTQHQSHPVT	87.5	87.5
(ii) Human		
<u>FcγRI vs FcγRII</u>		
CSG.MGKHRYTSAGIS : : CRGLFGSKNVSSSTVN	26.7	40
<u>FcγRI vs FcγRIII</u>		
CSG.MGKHRYTSAGIS : : CTGNIGY TLFSSHPVT	26.7	46.8
<u>FcγRII vs FcγRIII</u>		
CRGLFGSKNVSSSTVN : : CTGNIGYT LTFSSHPVT	37.5	43.8

4.7.2. Investigation of Fc γ R Expression by Flow Cytometry

Afferent lymph DC showed strong staining with each of the three rabbit antisera, with more than 70% of DC positive for fluorescence on staining with R220 FP1 antiserum. Affinity purified anti-peptide antibodies also stained a large proportion of DC, and control affinity purified antibodies gave a more discrete negative control peak than the preinoculation serum control. These results suggest the presence on DC of Fc γ R but do not distinguish between classes of Fc γ R. R220 serum also stained lymphocytes and neutrophils, neither of which express Fc γ RI in other species. Fc γ RII is found on B cells, while Fc γ RII and Fc γ RIII have been reported on subsets of T cells (Daeron et al 1988). Neutrophils express Fc γ RII and Fc γ RIII, although expression of Fc γ RI can be induced by IFN- γ (Van de Winkel & Anderson 1991). The reactivity of the serum with these cell types suggests a cross-reactivity between Fc γ RI, Fc γ RII and Fc γ RIII, as was also indicated by the results of Western blot analysis.

Surprisingly, the affinity purified anti-peptide antibodies did not stain afferent lymphocytes although the unfractionated serum did. In contrast, the staining of DC was improved by affinity purification. This could be due to the loss of certain anti-peptide antibodies during affinity purification, with high affinity antibodies retained on the peptide column, and may suggest structural differences in Fc γ R on DC and lymphocytes.

The R225 antiserum, raised against FP2, stained a large proportion of DC, again suggesting the expression of an Fc γ R by these cells. The R221 antiserum, raised against immunopurified ovine Fc γ R, stained DC, lymphocytes and neutrophils strongly. However, when anti-IgG activity was absorbed on an affinity column, the serum stained DC very weakly even when concentrated. Staining of lymphocytes was also considerably reduced. This may be due to loss of anti-Fc γ R antibodies during absorption on the affinity column. That the staining of DC observed with the unabsorbed R221 serum is likely to be due to the presence of the 40kD putative Fc γ R on the DC surface, rather than any anti-Ig activity in the serum, is demonstrated by

Western blot analysis of DC lysate (Section 4.5).

Changes in DC staining with the R220 antiserum after antigen challenge *in vivo* may reflect varying levels of Fc γ R expression by DC, although the antiserum would not distinguish between Fc γ RI and Fc γ RII, and the expression of these two molecules may be differentially regulated. The rabbit antiserum also contained anti-OVA antibodies, which could bind to OVA on the DC surface and increase the percentage of DC staining with rabbit antibodies. Removal of anti-OVA activity would be required to determine whether these antibodies have any effect on DC staining in these circumstances.

CHAPTER FIVE

EFFECT OF SPECIFIC ANTIBODY ON PBM PROLIFERATION

5. EFFECT OF SPECIFIC ANTIBODY ON PBM PROLIFERATION

5.1. Introduction

Previous work has shown the enhanced presentation of antigen by FcR-expressing APCs in the presence of specific antibody, by demonstrating potentiation of proliferative responses (Chapter 1). To determine whether this was also the case for ovine cells, experiments were carried out using unfractionated PBMs from sheep primed to OVA as both APCs and responding cells. For the enhanced presentation of antigen in immune complexes to occur, presenting cells must express Fc receptors. Monocytes, known to express Fc receptors for IgG in other species, are likely to act as APCs in these assays. Antigen-specific B cells are probably not present in sufficient numbers to activate T cells, and B cells present non-specific antigens poorly. Antibody also inhibits antigen presentation by specific B cells, with soluble antibodies competing with B cell mIg for antigenic epitopes (Lanzavecchia 1987). Human PBMs also contain a small percentage of DC (Thomas et al 1993); it is possible that DC contribute to antigen presentation in these assays.

5.2. Antibody Preparations

5.2.1. Antibody Activity and Isotype

Anti-OVA antibodies were affinity-purified from heat-inactivated antiserum. Activity of the anti-OVA antibodies was confirmed by ELISA using the serum of a primed animal as a positive control. The antibody isotype was also determined by ELISA, using second layer monoclonals specific for sheep Ig isotypes; all anti-OVA activity was of the IgG1 isotype. The concentration of the antibody preparation was calculated from OD readings at 280nm. The antibodies were dialysed into RPMI and filtered before use in assays.

The antibody/ antigen ratio for affinity purified antibodies and OVA was measured using Ouchterlony double gel diffusion. The equivalence point was taken as the

sharpest line of precipitation between wells of OVA and dilutions of specific antibody preparations. For the antibodies used in these experiments the antibody/ antigen ratio at equivalence was found to be 10:1.

An irrelevant affinity-purified antibody was required as a control for proliferation assays. Antibodies from the serum of a sheep primed to HSA were separated on an affinity column, and anti-HSA activity assessed by ELISA. No cross-reactivity of these antibodies against OVA was detected in an ELISA using OVA-coated plates (data not shown). The isotype of the anti-HSA antibody preparations was also IgG1.

5.2.2. $F(ab')_2$ Preparations

$F(ab')_2$ fragments of OVA-specific antibody were obtained by pepsin digestion of the intact antibody, followed by separation on an ion-exchange column. There was no residual whole IgG in these preparations, as determined by the absence of heavy chain on SDS-PAGE analysis (Fig.1, track 2). The other tracks show intact IgG (4), unseparated pepsin digest (3) and material eluted from the DEAE column with 0.3M NaCl (1). All of the residual heavy chain was present in the latter eluate. The anti-OVA activity of the $F(ab')_2$ antibody was evaluated by ELISA using a monoclonal specific for sheep Ig light chain as a second layer reagent. $F(ab')_2$ preparations had equivalent titres to the intact antibody from which they were prepared (for the sample used in these experiments both intact IgG and $F(ab')_2$ fragments had an anti-OVA titre of 1/1600).

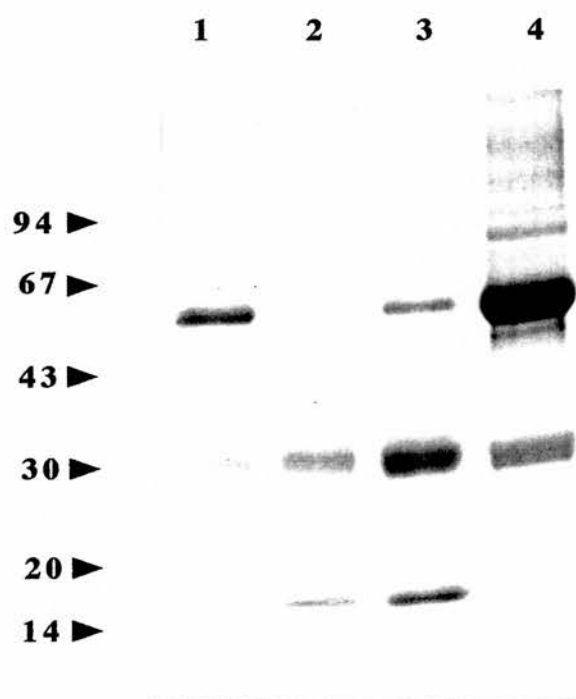
5.3. Effect of Specific Antibody on PBM Proliferation

Assays were set up as described in Chapter 2; 10^5 PBMs were plated per well, in a final volume of 200 μ l. After incubation at 37°C in 5% CO₂ for five days, PBM proliferation was determined by measurement of ³H-Thymidine incorporation over the last five hours of culture. The cultures were carried out in triplicate, with the results expressed as the arithmetic mean of cpm \pm SD.

Figure 1

Pepsin digestion of affinity purified anti-OVA antibodies was carried out in order to obtain $F(ab')_2$ fragments. After ending the reaction and dialysis into 10mM phosphate buffer, the antibody was passed through an ion-exchange column to separate the $F(ab')_2$ portions from residual intact antibody. The $F(ab')_2$ antibody was expected to pass straight through the column, while the intact antibody was retained and subsequently eluted with sodium chloride solution. These two antibody samples, with unseparated antibody after pepsin digestion, and the original affinity purified preparation, were analysed by SDS-PAGE on a 5-25% gradient slab gel. The gel was silver-stained.

FIGURE 1 SDS-PAGE Analysis of A (Fab')₂ Preparation
of Anti-OVA Antibodies



1 - Material eluted from DE column

2 - F(ab')₂ preparation

3 - Unseparated anti-OVA antibodies after pepsin digestion

4 - Anti-OVA antibodies before digestion

The proliferation of PBMs to OVA at suboptimal concentrations was enhanced considerably by the addition of specific affinity purified antibodies (Fig.2). This increase in proliferation was seen over a range of OVA dilutions but was greatest at suboptimal concentrations. In the experiment shown in Fig.2, proliferation was increased a maximum of 3.4 times above that seen with OVA alone. No significant enhancement was seen at an OVA concentration of $100\mu\text{gml}^{-1}$, where PBMs proliferated optimally to OVA alone. When cultured with anti-OVA antibody alone, the PBMs did not show any proliferative response above that seen with medium only. Similar results, showing potentiation of the PBM response to suboptimal concentrations of OVA on the addition of specific antibody, were obtained with five sheep (Table 1). The changes in proliferation of PBMs observed on the addition of specific antibody were statistically significant in each case, and ranged from a 1.8-fold (sheep 23) to a 5.8-fold (sheep 82) increase. In each case, no proliferation over that seen with medium only occurred where PBMs were cultured with anti-OVA antibody alone.

5.4. Effect of F(ab')_2 Anti-OVA Antibody on PBM Proliferation

F(ab')_2 fragments of anti-OVA antibody were used in place of intact antibodies in one experiment. The F(ab')_2 antibodies did not cause any significant enhancement of the PBM proliferation to OVA (Fig.3) although they retained anti-OVA activity as detected by ELISA (section 5.2.). The requirement for an intact Fc portion of specific antibody suggests the involvement of $\text{Fc}\gamma\text{R}$ in the enhanced PBM response.

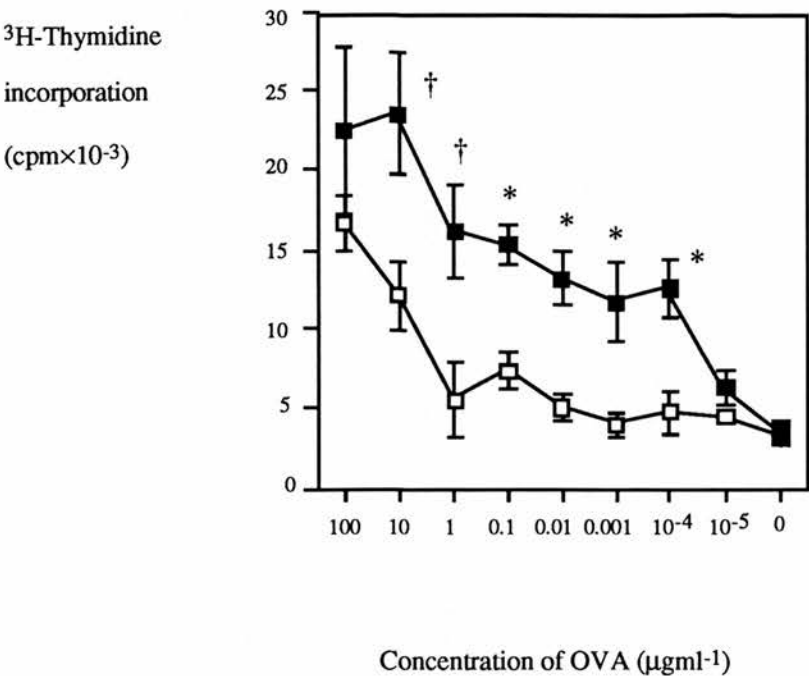
5.5. Effect of Irrelevant Antibody on PBM Proliferation

Irrelevant affinity purified antibodies were used as a control in two assays with PBMs. A range of OVA dilutions was used, and specific or anti-HSA antibodies were added at the same concentration. The activity of HSA-specific antibodies was confirmed in an ELISA with HSA-coated plates before use (data not shown). While specific antibody significantly increased the proliferation of PBMs to OVA, irrelevant antibody did not

Figure 2

10^5 PBMs from a primed sheep were cultured with dilutions of OVA, with and without specific antibody at $30\mu\text{gml}^{-1}$. After five days of culture, PBM proliferation was assessed by measurement of ^3H -Thymidine incorporation over the final five hours. The results are the mean cpm of triplicate cultures. Statistical significance was determined using a Two Sample T Test.

FIGURE 2 PBM Proliferation To OVA is Enhanced by Specific Antibody



Key

- OVA only
- OVA and anti-OVA antibody
- † $p < 0.05$
- * $p < 0.001$

Table 1

PBMs from sheep primed to OVA were cultured with OVA at a concentration of $0.1\mu\text{gml}^{-1}$. Affinity purified anti-OVA antibody was added to some wells at a concentration of $30\mu\text{gml}^{-1}$. Controls were DC and T cells cultured with medium only, or with anti-OVA antibody only. The antibody and antigen dilutions were added to the wells first and incubated at 37°C for 30 minutes to allow the formation of immune complexes. PBM proliferation was assessed by measurement of ^3H -Thymidine incorporation over the last five hours of culture. The results shown are the mean cpm of triplicate cultures \pm SDs. The statistical significance of the enhancement which occurred on the addition of specific antibody was determined using a Two Sample T Test.

TABLE 1 PBM Proliferation to OVA is Enhanced by Specific Antibody

PBM Proliferation [³ H-Thymidine Incorporation (cpm)]					
Sheep Number	OVA only	OVA and anti-OVA antibody	Fold Enhancement With Antibody	Medium only	Anti-OVA antibody only
77	9218 ± 921	31453 ± 4251*	3.4	2302 ± 234	3254 ± 682
151	15696 ± 2010	62266 ± 6098*	4	1830 ± 171	1860 ± 136
21	5510 ± 2311	22399 ± 3247*	4.1	3217 ± 636	3296 ± 1211
82	4687 ± 814	27092 ± 3300*	5.8	1883 ± 926	1689 ± 872
23	15510 ± 1178	27331 ± 1767 †	1.8	2651 ± 478	2249 ± 592

† p < 0.05

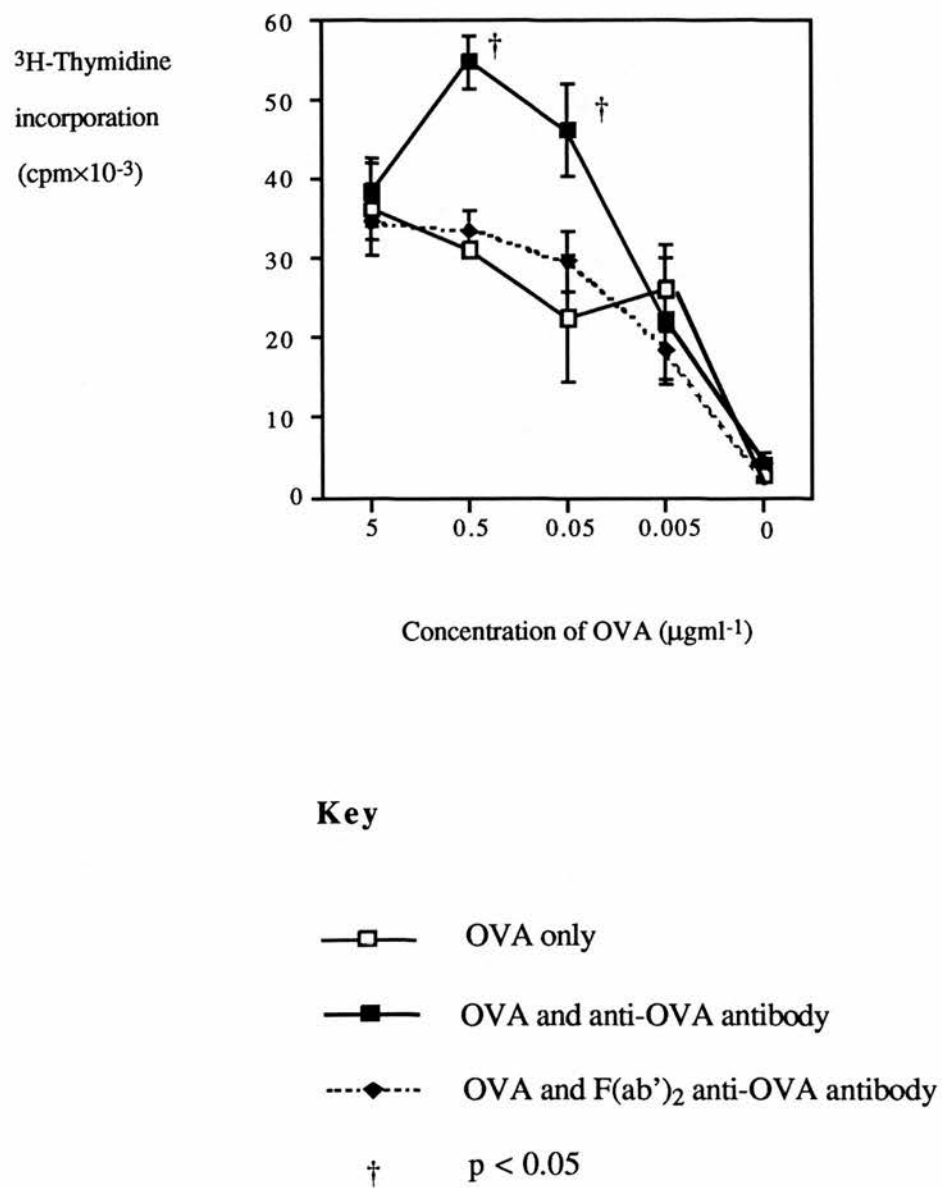
* p < 0.001

for the difference between proliferation to OVA only and that to OVA and antibody

Figure 3

PBMs from a sheep primed to OVA were cultured with dilutions of OVA alone, or with added intact specific antibody at $30\mu\text{gml}^{-1}$, or F(ab')_2 portions of specific antibody at the same concentration. The antibody and antigen dilutions were incubated at 37°C for 30 minutes to allow the formation of immune complexes before the cells were added in a final volume of $200\mu\text{l}$. The cells were cultured for five days before addition of ^3H -Thymidine over the last five hours. The results are the mean cpm of triplicate cultures \pm SDs. Statistical analysis was carried out using a Two Sample T Test.

FIGURE 3 An Intact Fc Portion of Specific Antibody
Is Required For Enhanced PBM Proliferation



(Fig.4). In fact, in one of the two experiments (sheep 151), anti-HSA antibody at the concentration used decreased the proliferative response of PBMs to OVA. This inhibition was statistically significant at the two points shown, as determined using a Two Sample T Test. In the other experiment (sheep 77) the anti-HSA antibody had no significant effect on the proliferation of PBMs to OVA.

5.6. Effect of Anti-Class II Antibody on PBM Proliferation

Exogenous antigens such as OVA are usually processed for presentation as peptides on MHC class II molecules, for interaction with CD4 T cells (Unanue 1984). An anti-MHC class II monoclonal antibody was included in a PBM assay to determine whether the enhanced proliferation seen with OVA and specific antibodies involved MHC class II. SW73.2 was used at $20\mu\text{gml}^{-1}$ and was present throughout the culture period (Fig.5). A control antibody, rat anti-mouse IgG, was used at the same concentration. The proliferation of PBMs to OVA and specific antibody was significantly reduced on the inclusion of SW73.2, while the control antibody had no significant effect.

5.7. Effect of Altering the Antibody/ Antigen Ratio on PBM Proliferation

The antigen/ antibody ratio affects the degree of enhancement of proliferation of responding cells when antigen is presented by murine macrophages (Manca et al 1991). In these experiments, the maximum enhancement of proliferation with specific antibody occurred at moderate antibody excess in all five sheep. OVA was used at a concentration of $0.1\mu\text{gml}^{-1}$, with anti-OVA antibody at $30\mu\text{gml}^{-1}$, to give a 30-fold antibody excess (Fig.6 (i)). PBM proliferation on the addition of specific antibody increased from 1.8 to 5.8 times with these antibody and antigen concentrations. Significant increases in PBM proliferation were observed in two of four sheep with OVA at a concentration of 10 ngml^{-1} and anti-OVA antibody at $30\mu\text{gml}^{-1}$, representing a 300-fold antibody excess (Fig.6 (ii)). At a 3000-fold antibody excess, with OVA at 1 ngml^{-1} , significant enhancement of PBM proliferation on addition of specific antibody

Figure 4

10⁵ PBMs from each of two primed sheep were cultured with varying dilutions of OVA alone, or with added specific antibodies at 30µgml⁻¹, or anti-HSA antibodies at 30µml⁻¹. Controls included cells incubated with each antibody alone, and medium only. PBM proliferation was assessed after a five day culture period by measuring the incorporation of ³H-Thymidine. The results are the mean cpm of triplicate cultures ± SDs. The statistical significance of the difference between the response to OVA alone and proliferation to OVA with added antibody was determined using a Two Sample T Test.

FIGURE 4 PBM Proliferation is Not Enhanced
by Irrelevant Antibody

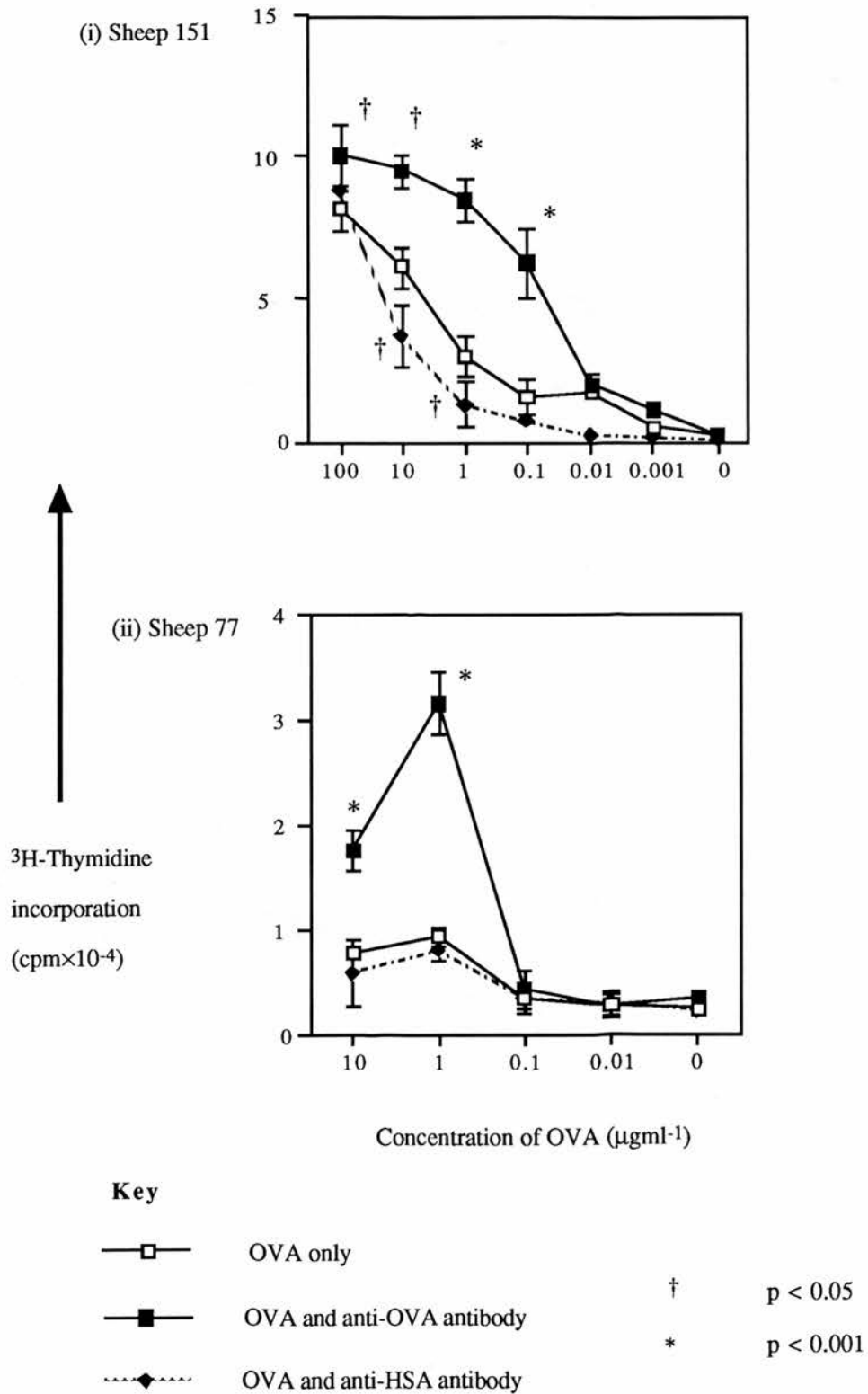


Figure 5

10^5 PBMs were cultured with

- (i) OVA dilutions
- (ii) OVA and specific antibody at $30\mu\text{gml}^{-1}$
- (iii) OVA, specific antibody and SW73.2 anti-class II antibody at $20\mu\text{gml}^{-1}$.
- (iv) OVA, specific antibody and rat anti-mouse IgG control antibody at $20\mu\text{gml}^{-1}$

Proliferation assays were carried out over a five day period. The results are the mean cpm of triplicate cultures \pm SDs. The statistical significance was determined using a Two Sample T Test. The significance values shown for the proliferation to OVA and specific antibody refer to the difference between the response of PBMs to OVA only and to OVA and anti-OVA antibody. Those for the cultures including SW73.2, indicate the significance of the difference between the proliferation to OVA and anti-OVA antibody and that to OVA, anti-OVA antibody and SW73.2 antibody.

FIGURE 5 The Enhanced Proliferation of PBMs to OVA and Anti-OVA Antibody is Inhibited by Anti-MHC Class II Antibody

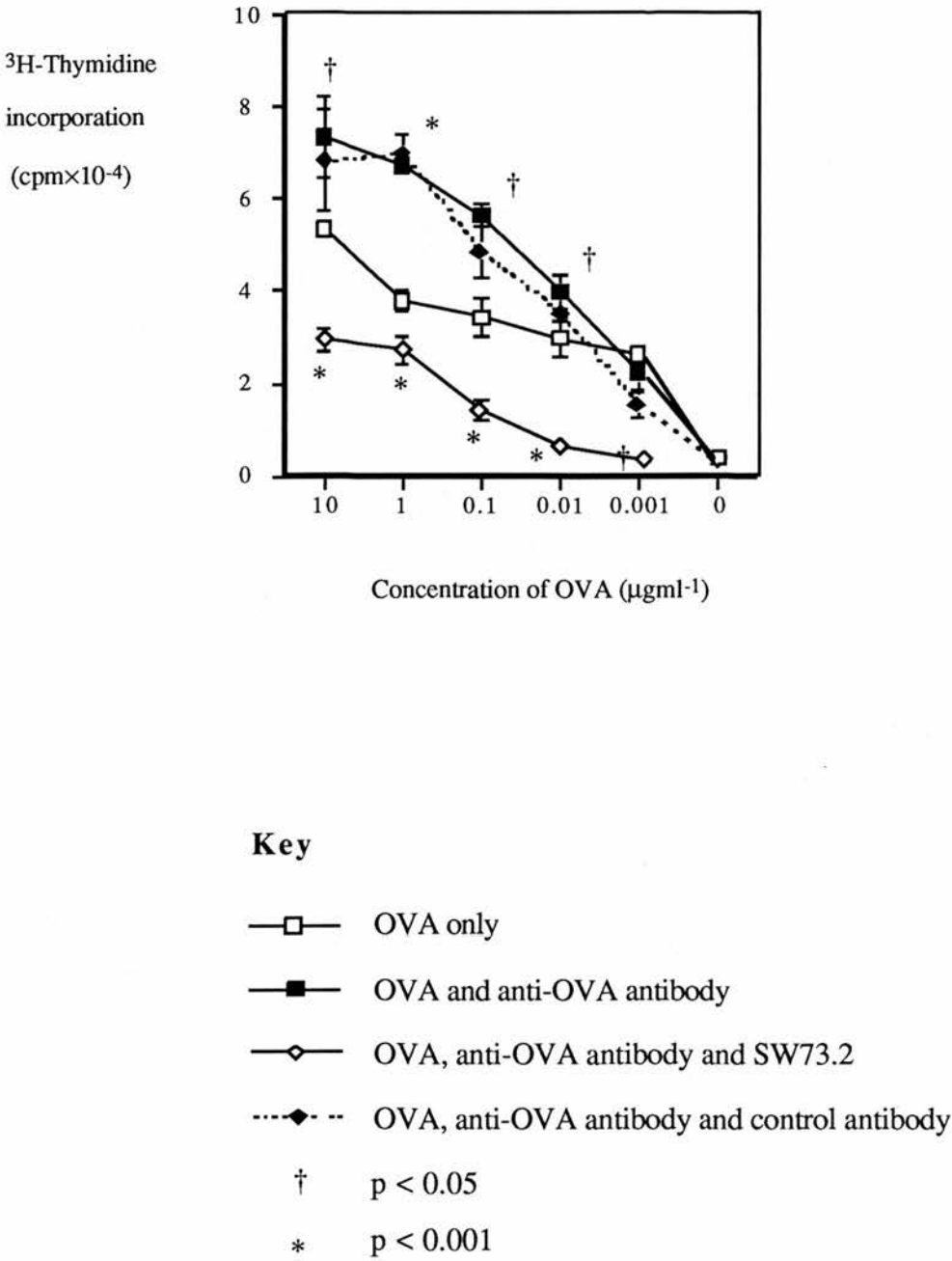
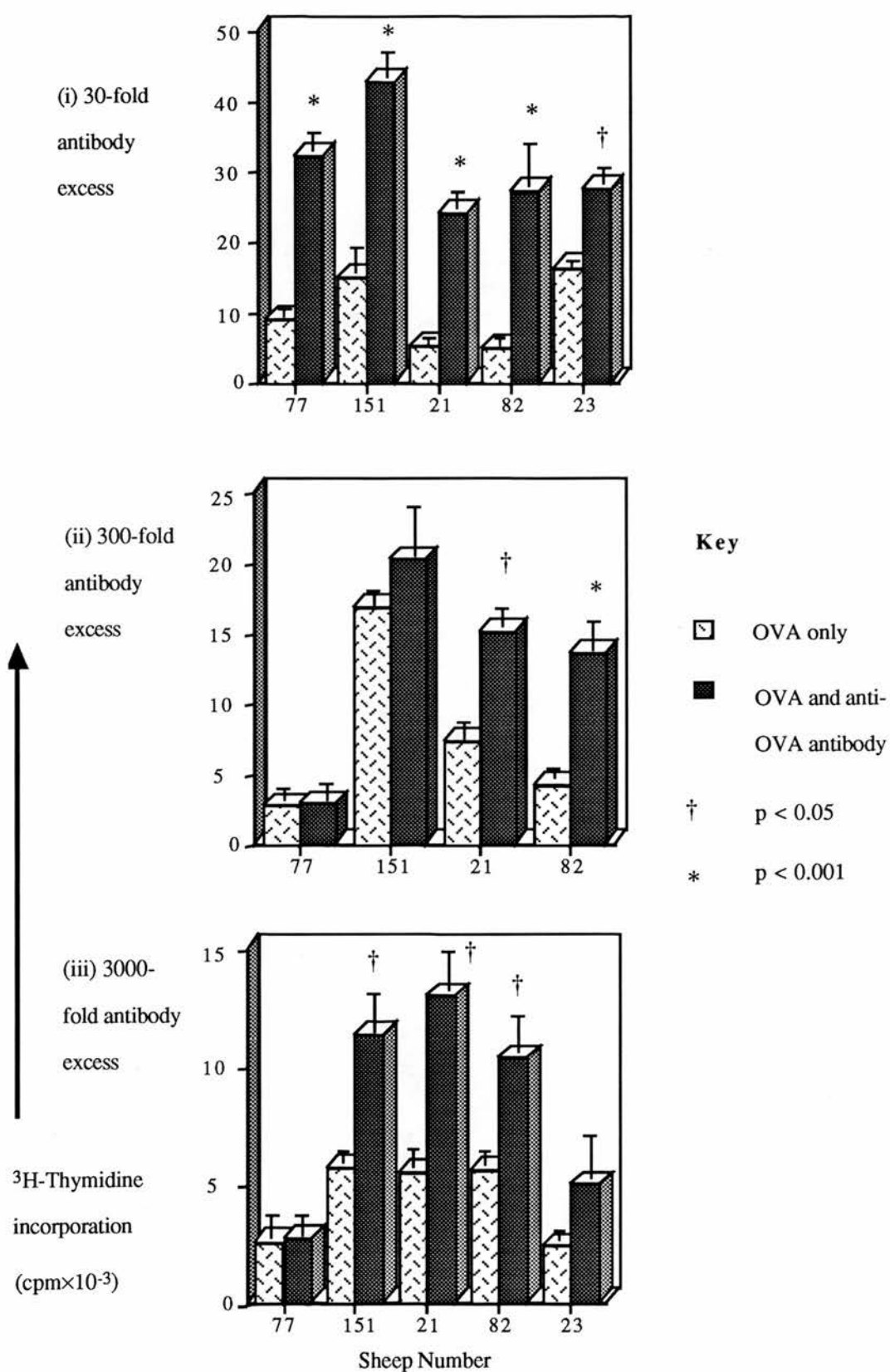


Figure 6

OVA and specific antibody dilutions were added to 96 well plates and incubated for 30 minutes at 37°C to allow the formation of immune complexes. 10^5 PBMs from a primed sheep were added and incubated for five days before measurement of ^3H -Thymidine uptake over the last five hours of culture. In (i) the PBM proliferation to OVA at $0.1\mu\text{gml}^{-1}$ is shown with and without specific antibody at $30\mu\text{gml}^{-1}$, representing a 30-fold antibody excess. In (ii), the OVA concentration is 10 ngml^{-1} with specific antibody at $30\mu\text{gml}^{-1}$, representing a 300-fold antibody excess, and in (iii) the OVA concentration is 1 ngml^{-1} with specific antibody at $30\mu\text{gml}^{-1}$, representing a 3000-fold antibody excess. The controls for this experiment included PBMs cultured with medium only, or with anti-OVA antibody only. The results are the mean cpm of triplicate cultures \pm SD; statistical significance was determined using a Two Sample T Test.

FIGURE 6 Effect of Antibody/ Antigen Ratio on PBM Proliferation



were observed in three of five sheep (Fig.6 (iii)). Here, the maximum increase in the PBM response occurred in sheep 21, where a 2.4-fold enhancement was observed when specific antibody was included. The PBMs from sheep 23 showed some increase in proliferation to OVA on addition of antibodies at extreme excess, but this was not significant. No increase was seen at these concentrations with the PBMs from sheep 77.

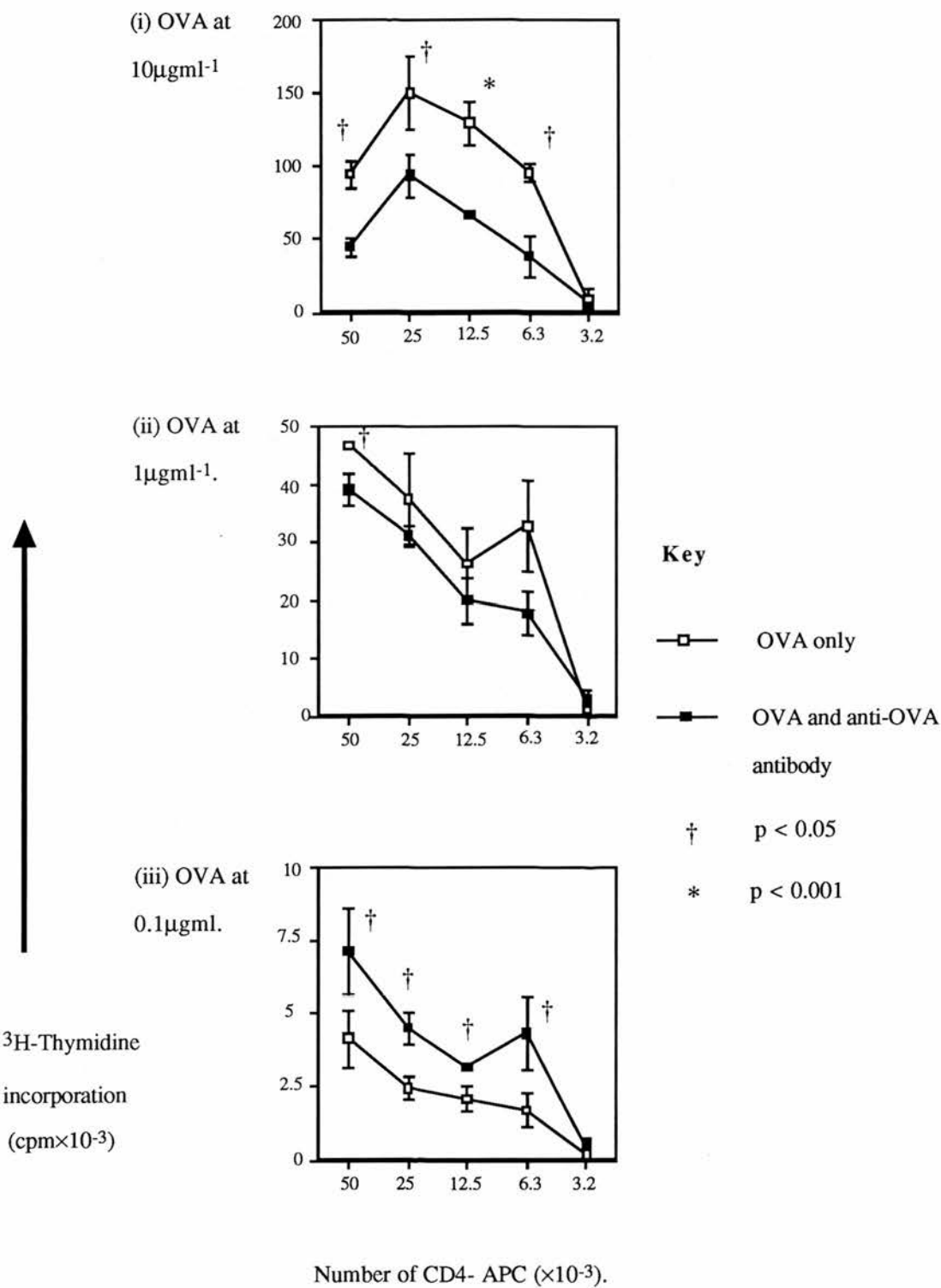
To investigate the effect of antigen excess on PBM proliferation, CD4⁺ T cells were set up in culture with antigen/ antibody complexes at ratios of 3.3-fold antigen excess, or 3 or 30-fold antibody excess. CD4⁺ T cells were separated from the PBMs of a sheep primed to OVA on a MACS as described in Chapter 2. The unstained, CD4⁻ population of cells which came straight through the magnetised column was also collected and used as a source of APCs after irradiation with 2500rads. A titration of CD4⁻ APCs was added to 10⁵ CD4⁺ T cells per well, with three concentrations of OVA and a fixed specific antibody concentration (Fig.7).

Where OVA was used at 10 μ gml⁻¹ (Fig.7(i)), i.e. at 3.3-fold antigen excess, specific antibody inhibited the proliferative response of CD4⁺ T cells to OVA. The inhibition of proliferation on the addition of antibody was found to be statistically significant using a Two Sample T Test. Anti-OVA antibody alone had no effect on PBM proliferation (data not shown). In (ii), the OVA concentration was 1 μ gml⁻¹ and anti-OVA antibody added at 30 μ gml⁻¹ inhibited the proliferation of PBMs to OVA to a lesser extent. Indeed, the decrease in the CD4⁺ T cell response when anti-OVA antibody was added was significant at only one point. The latter concentration of antibody and antigen represented a 3-fold antibody excess, and in the assays with unseparated PBMs specific antibody enhanced proliferation to OVA at this ratio (Fig.2). The proliferation of CD4⁺ T cells to OVA at 0.1 μ gml⁻¹ was significantly enhanced by the addition of anti-OVA antibodies at 30 μ gml⁻¹, giving a 30-fold antibody excess (Fig.7 (iii)). The antigen/ antibody ratio is clearly important in these assays, as in the assays with unfractionated PBMs. However, inhibition of proliferation to OVA was not observed on the inclusion of anti-OVA antibodies in assays using unfractionated PBMs.

Figure 7

CD4⁺ and CD4⁻ cells were obtained from the PBMs of an OVA-primed sheep by separation on a MACS. 10⁵ CD4⁺ cells were cultured with varying numbers of irradiated CD4⁻ cells, with OVA only or OVA and specific antibodies.. In (i) OVA was used at 10µgml⁻¹, in (ii) at 1µgml⁻¹ and in (iii) at 0.1µgml⁻¹. Anti-OVA antibodies were included in some wells at 30µgml⁻¹. The cells were incubated at 37°C for five days; uptake of ³H-Thymidine was measured over the last five hours of culture. The results are the mean cpm of triplicate cultures ± SDs. The statistical significance was determined with a Two Sample T Test..

FIGURE 7 Effect of Anti-OVA Antibody on Proliferation of CD4+ T Cells



5.8. Discussion

The proliferation of PBMs from primed sheep to substimulatory concentrations of OVA was greatly enhanced in the presence of specific antibodies, although antibody alone had no effect. A significant increase in PBM proliferation on the addition of anti-OVA antibodies was observed in experiments with five sheep, and the greatest increase in the PBM response occurred at suboptimal concentrations of OVA.

Intact specific antibody was necessary for potentiation of the proliferative response, with $F(ab')_2$ fragments being completely ineffective despite their retention of anti-OVA antibody activity. An Fc portion of antibody was therefore required, presumably for interaction with Fc receptors for IgG on the surface of the APC.

The requirement for specific antibodies capable of forming immune complexes with OVA was demonstrated by the inclusion of irrelevant affinity purified antibodies at the same concentration. Anti-HSA antibody was of the same isotype as the anti-OVA antibodies (IgG1) and was active against HSA when tested by ELISA, but failed to increase PBM proliferation to OVA. Indeed, the irrelevant antibody actually inhibited the PBM response to OVA to a significant degree in the PBMs from one of the two animals used (Fig.4). The mechanism by which inhibition occurs is not clear; OVA, in the absence of specific antibodies, is probably taken up by pinocytosis and it is difficult to see what effect non-specific antibodies would have on this. Uncomplexed anti-HSA antibodies should not bind to $Fc\gamma RII$, but could bind to $Fc\gamma RI$. However, binding of IgG to $Fc\gamma RI$ provides an activational stimulus to the cell, and therefore should not cause the inhibitory effect observed. $Fc\gamma RI$, with its high affinity for monomeric IgG, should also be saturated with IgG under physiological conditions (Van de Winkel & Anderson 1991).

The enhanced proliferation of PBMs to OVA-anti-OVA complex was significantly inhibited by the inclusion of anti-MHC class II antibodies in the cultures. The presentation of at least a proportion of antigen taken up via $Fc\gamma R$ on the APC surface was therefore dependent on an MHC class II pathway. $CD4^+$ T cells, which recognise

peptide antigens in the context of MHC class II molecules on the APC surface, are likely to be the main responding cells in the PBM population. Some proliferation by CD8+ cells and B cells may also be taking place.

The degree of increase in PBM proliferation on the addition of specific antibody was dependent on the antibody/ antigen ratio, and was greatest in moderate (30-fold) antibody excess. However, significant enhancement was also apparent at 300-fold antibody excess in two of four sheep and at 3000-fold antibody excess in three of the five sheep.

PBMs contain several cell types capable of acting as APCs. For the experiments described here, APC requirements include the expression of Fc γ R, the ability to take up and process antigen in complex form and to present peptides to responding T cells. Monocytes are non-specific Fc γ R-expressing accessory cells, while B cells are efficient APCs for specific antigen acquired via membrane Ig receptors. DC are also present in the PBM population in low numbers; DC from human blood have recently been reported to express Fc γ RI and Fc γ RII, and comprise 2-3% of PBMs (Thomas et al 1993). Although B cells also express Fc receptors for IgG, they are unlikely to play a part in the enhancement of proliferation when specific antibody at excess and low levels of antigen are present. While immune complex attachment to Fc γ RII provides stimulatory signals to cells such as monocytes, cross-linkage of Fc γ RII and mIg on the B cell surface delivers an inhibitory signal to the B cell (Klaus et al 1987). The murine macrophage Fc γ RII is internalised on ligand binding while the B cell isoform remains on the cell surface (Miettinen et al 1989). This mechanism has been proposed as a means of preventing B cells from taking up immune complexes and acting as non-specific APCs (Lanzavecchia 1990). Monocytes (and possibly DC) are therefore more likely to be functioning as APCs in these proliferation assays.

In order to separate the APC and responder cell populations, one experiment was carried out using irradiated CD4- cells obtained from PBMs as APCs, with the positively selected CD4+ T cells acting as responders. The proliferation of CD4+ cells

to OVA was actually inhibited by specific antibody in antigen excess (Fig.7(i)) and, to a lesser extent, at moderate (3-fold) antibody excess (Fig.7(ii)). A further ten-fold antigen dilution was necessary before specific antibody increased proliferation to antigen alone (Fig.7(iii)). This last concentration represented a 300-fold antibody excess. These results are in contrast to the PBM assays, in which, although maximum enhancement of proliferation was observed at moderate (3 to 30-fold) antibody excess, significant inhibition of proliferation to OVA on the inclusion of specific antibody did not occur.

To assess the relative contributions of monocytes and DC in the PBM population to antigen presentation in these assays, purification of each cell type would be required. However, purification procedures may involve culture and techniques which modify the ability of the selected cells to present antigen and elicit a proliferative response from T cells. A rapid isolation procedure with minimal culture steps would thus be necessary to obtain unaltered DC and monocytes from fresh PBMs.

CHAPTER SIX

THE EFFECT OF SPECIFIC ANTIBODY ON ANTIGEN PRESENTATION BY DC

6. THE EFFECT OF SPECIFIC ANTIBODY ON ANTIGEN PRESENTATION BY DC

6.1. Introduction

DC are known to be potent accessory cells for the induction of T cell responses. The mechanisms involved in antigen uptake and processing by DC remain to be established; DC are poorly phagocytic and the expression of Fc receptors for immunoglobulins is variable depending on the source of the cells.

DC from sheep afferent lymph show greatly enhanced uptake of antigen in the presence of specific antibody (Harkiss et al 1990); this increase is dependent on the presence of an intact Fc portion of IgG, and varies with the antigen/ antibody ratio. DC are also capable of rosette formation with IgG-coated erythrocytes; these data imply that DC express Fc γ R which are involved in the efficient uptake of immune complexes. The results in Chapters 3 and 4 demonstrated DC staining with antisera raised against peptides of extracellular domains of an Fc receptor for IgG, and also with an antiserum raised against a putative ovine Fc γ R. Western blot analysis demonstrated the presence on DC of molecules of suitable size for Fc γ RI and Fc γ RII.

The functional significance of the enhanced uptake of antigen by DC in the presence of antibody is unclear. Enhanced presentation of antigen in the form of immune complexes has been shown for other APCs (see Introduction), and for unfractionated ovine PBMs (Chapter 5), but not for afferent lymph DC. The experiments described in this chapter were carried out to investigate the role of Fc receptors on DC in the presentation of antigen to T cells. DC and T cells were obtained from sheep primed to OVA and cultured *in vitro* with OVA only or OVA and specific antibodies. Proliferation of T cells was used to measure antigen presentation by DC.

6.2. Purity of CD4+ Responder T Cells

CD4+ T cells at > 93% purity were obtained from PBMs of primed sheep by selection

on a MACS as described in Chapter 2 (Fig.1). The purified CD4+ T cells were cultured with OVA or OVA with specific antibodies to assess possible contamination with APCs. No proliferation by CD4+ T cells above that with medium alone was seen in any experiment unless DC were added.

6.3. Specific Antibody Enhances CD4+ T Cell Proliferation

For these experiments OVA and affinity-purified antibodies at varying concentrations were added to plates for incubation at 37°C to allow immune complex formation before the addition of DC and T cells. T cell proliferation in a representative assay is shown in Fig 2. The cultures were carried out in triplicate with results expressed as the arithmetic mean of counts per minute (cpm) +/- standard deviation (SD).

The proliferation of CD4+ T cells to suboptimal concentrations of OVA was greatly enhanced by the addition of anti-OVA antibodies. The increased proliferation required the presence of both OVA and specific antibodies; no proliferation above background occurred when DC and T cells were cultured with antibody alone (Table 1 & 2). Both DC and CD4+ T cells were required for proliferation to occur; control wells with DC or T cells only cultured with medium, OVA or OVA and anti-OVA antibody never gave counts of >300 (Table 1 and data not shown), indicating that T cells were not contaminated with APCs and DC were not contributing to the proliferation observed.

This enhancement was observed in experiments with six sheep, with the effect of specific antibody on the T cell proliferation to OVA ranging from a 5-fold to a 203-fold increase (Table 2). The enhancement was found to be highly significant in each case, using a Two Sample T Test. Irrelevant affinity purified antibodies specific for HSA were included in experiments with two sheep, and had no stimulatory effect on T cell proliferation at suboptimal concentrations of OVA. Representative results are shown in Fig.3.

Figure 1

PBMs were stained with biotinylated anti-CD4 monoclonal antibody followed by streptavidin-labelled magnetic microbeads. Streptavidin-PE was also added at this stage to enable assessment of the purity of the CD4+ selected population by flow cytometry. The PBMs were then separated on a MACS. FACS profiles for positively selected cells (i) and unselected cells which passed through the magnetised column (ii) are shown. The percentage of cells positive for fluorescence and therefore CD4 expression was 94% in (i) and 5% in (ii).

FIGURE 1 CD4 Expression of Cells Separated by MACS

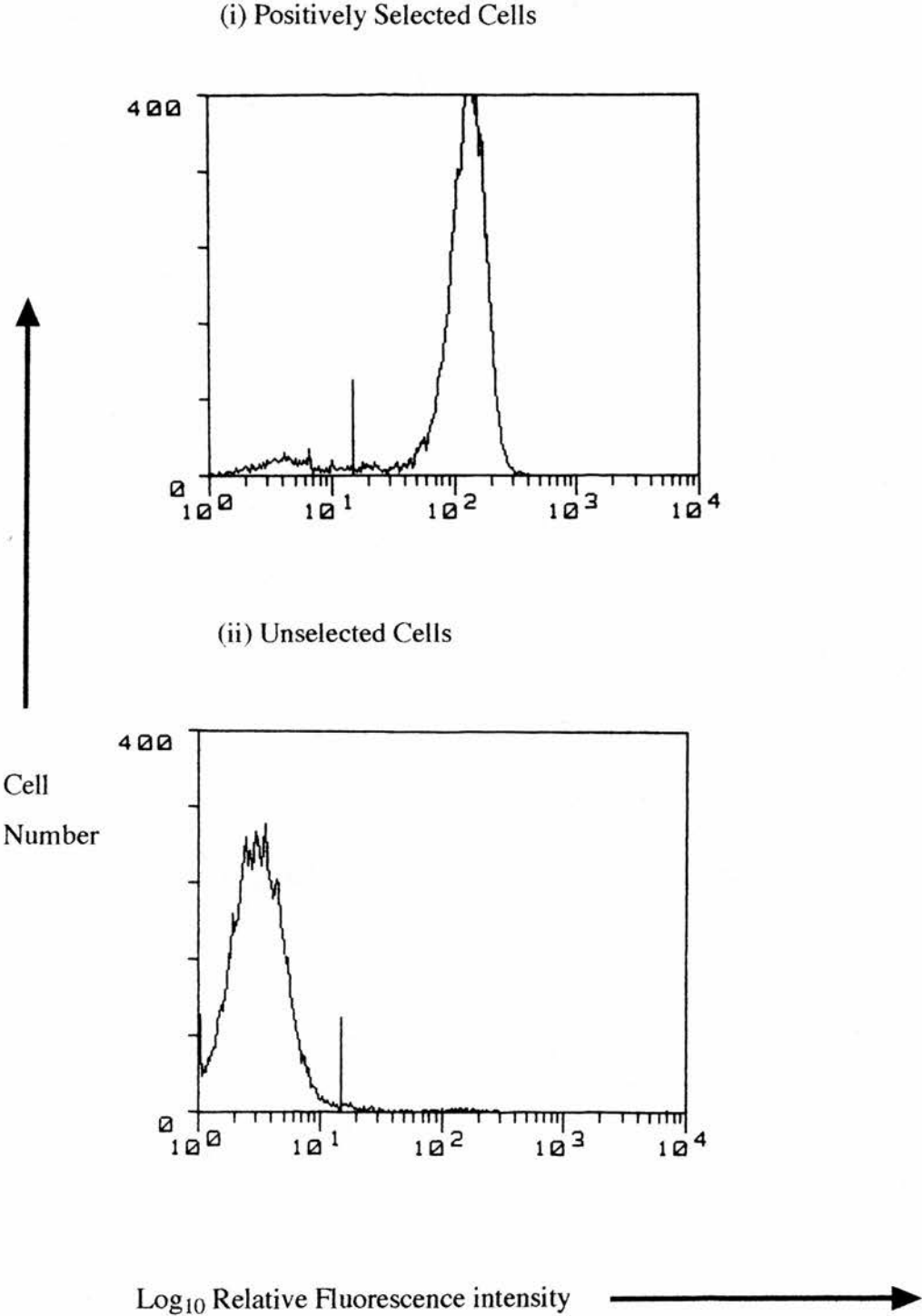
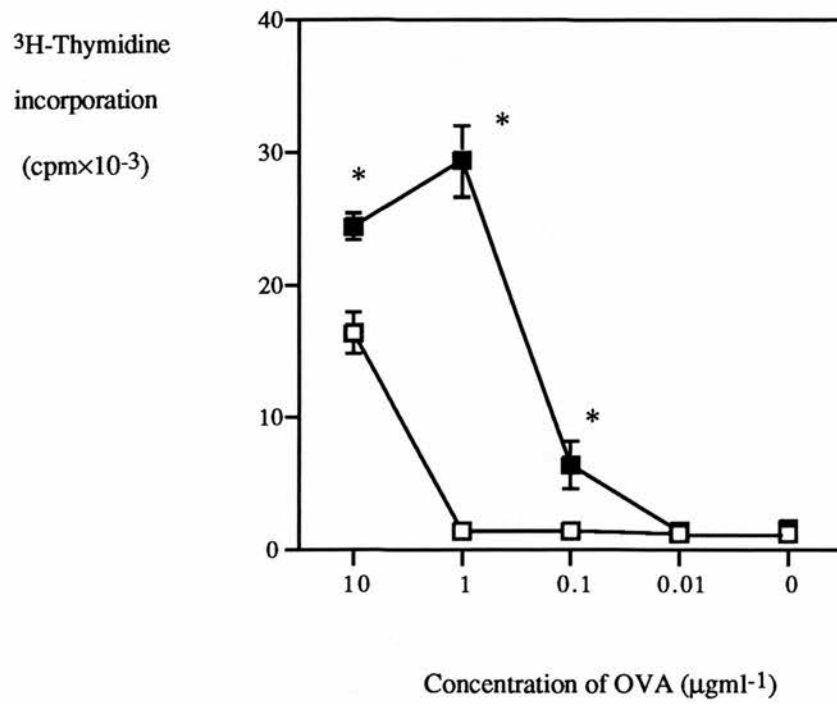


Figure 2

DC and CD4⁺ T cells from sheep primed to OVA were purified as described in Materials and Methods. 5×10^4 DC and 10^5 T cells were cultured with dilutions of OVA in 200 μ l volumes in 96-well plates. Specific antibody was included in some wells at a final concentration of 30 μ gml⁻¹. Antibody and antigen were added to the wells first; incubation at 37°C for 30 minutes allowed the formation of immune complexes before addition of DC and T cells. After a five day culture period, T cell proliferation was assessed by measurement of ³H-thymidine incorporation. The results are expressed as the mean counts of triplicate cultures \pm standard deviations (SD). Significance values were calculated using a Two Sample T Test.

FIGURE 2 Specific Antibody Enhances T Cell Proliferation to OVA



Key

—□— OVA only

—■— OVA and anti-OVA antibody

* $p < 0.001$

Table 1

DC and CD4+ T cells from an OVA-primed sheep were cultured with one of the following:- OVA at $1\mu\text{gml}^{-1}$; anti-OVA antibodies at $30\mu\text{gml}^{-1}$; both OVA and antibodies; or medium only. Each cell type was also cultured separately with the range of additions as shown. The results are the mean cpm of triplicate cultures \pm SDs, and illustrate proliferation in a representative experiment.

TABLE 1 Proliferation Assay Controls

Cells	Cultured With	Mean cpm \pm SD
DC + T	OVA	17862 \pm 3026
DC + T	OVA + Ab	143106 \pm 10500
DC + T	Ab	288 \pm 29
DC + T	Medium	521 \pm 79
DC	OVA	125 \pm 35
DC	OVA + Ab	198 \pm 32
DC	Ab	226 \pm 72
DC	Medium	113 \pm 6
T	OVA	251 \pm 36
T	OVA + Ab	140 \pm 14
T	Ab	188 \pm 40
T	Medium	119 \pm 5

Table 2

DC and CD4+ T cells from sheep primed to OVA were cultured with OVA only at $0.1\mu\text{gml}^{-1}$, or OVA at $0.1\mu\text{gml}^{-1}$ and anti-OVA antibodies at $30\mu\text{gml}^{-1}$. The antibody and antigen dilutions were added to the wells first and incubated at 37°C for 30 minutes to allow the formation of immune complexes. DC and CD4+ T cells were then added, and cultured at 37°C for five days. T cell proliferation was assessed over the last five hours of culture, by measurement of ^3H -Thymidine incorporation . The results are the mean cpm of triplicate cultures \pm SDs. The statistical significance of the difference between the T cell response to OVA alone and that to OVA and specific antibodies was calculated using a Two Sample T Test.

TABLE 2 Specific Antibody Enhances T Cell Proliferation to OVA at Suboptimal OVA Concentrations

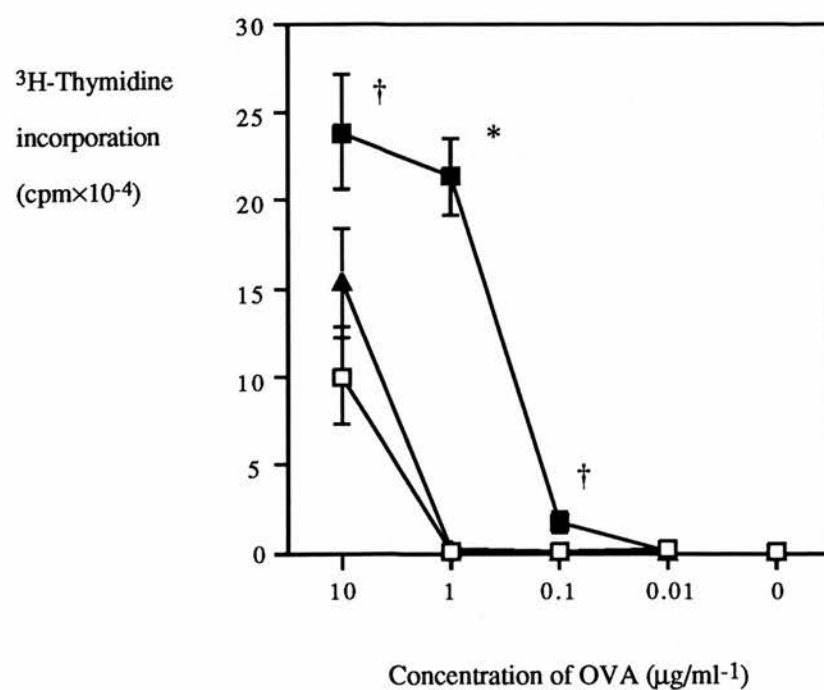
Sheep Number	T Cell Proliferation [³ H-Thymidine Incorporation (cpm)]			
	OVA only	OVA and anti- OVA antibody	Fold Enhancement with Antibody	Anti-OVA Antibody only
150	1538 ± 507	27669 ± 2323 [*]	18	1037 ± 360
151	1459 ± 94	36139 ± 3900 [*]	24.8	521 ± 79
156	363 ± 30	61505 ± 6131 [*]	169.4	102 ± 83
21	1621 ± 344	12261 ± 541 [*]	7.6	1654 ± 272
19	1216 ± 327	11500 ± 1652 [*]	9.5	1354 ± 421
23	3353 ± 438	16875 ± 1616 [*]	5	1204 ± 827
				2634 ± 602

^{*} p < 0.001 for the difference between proliferation to OVA only and that to OVA and antibody

Figure 3

Purified DC and T cells were added to wells containing OVA only, OVA and specific antibody at $30\mu\text{gml}^{-1}$, or OVA and anti-HSA antibody at $30\mu\text{gml}^{-1}$ (see key). Both antibody preparations were affinity purified from sera of primed sheep, and were of the same (IgG1) isotype. Anti-HSA antibodies showed no cross-reactivity with OVA when tested by ELISA. Controls included DC and T cells cultured with anti-HSA or anti-OVA antibody alone. Results are the means of triplicate counts; standard deviations are shown by the error bars. Statistical analysis was carried out using a Two Sample T Test and significant differences in the proliferation of T cells are shown.

FIGURE 3 Irrelevant Antibody Does Not Potentiate
T Cell Proliferation to OVA



Key

- OVA only
- ▲— OVA and anti-HSA antibody
- OVA and anti-OVA antibody

\dagger $p < 0.05$

$*$ $p < 0.001$

6.4. Effect of F(ab')₂ Antibody on T Cell Proliferation

F(ab')₂ fragments of specific antibody were used to investigate the role of DC Fc receptors for IgG in the potentiation of T cell responses. While F(ab')₂ preparations retained anti-OVA antibody activity when tested by ELISA, they were completely ineffective in these assays and did not increase CD4⁺ T cell proliferation above that seen with OVA alone. This experiment was carried out with two sheep and the results are shown in Fig.4.

6.5. Effect of Anti-MHC Class II Antibody on T Cell Proliferation

The presentation of antigen to CD4⁺ T cells involves peptides in the antigen-binding grooves of MHC class II molecules on the DC surface interacting with the antigen-specific TCR of the T cell. To determine whether the enhancement of T cell stimulation that occurred with specific antibody was dependent on class II molecules, SW73.2, a rat monoclonal with specificity for ovine MHC class II, was included in some wells throughout the culture period of one experiment. Complete inhibition of proliferation to OVA plus antibody occurred on addition of SW73.2 at 20 µgml⁻¹, while the control monoclonal (rat anti-mouse IgG), used at the same Ig concentration, had no significant inhibitory effect (Fig 5).

6.6. Effect of Altering the Antigen/ Antibody Ratio on T Cell Proliferation

The degree of enhancement of antigen uptake in the presence of specific antibody is dependent on the antigen/ antibody ratio. Experiments were carried out with fixed, suboptimal concentrations of OVA and added specific antibody in varying amounts to investigate the role of the antigen/ antibody ratio in functional assays.

The extent of the increase in T cell proliferation seen in this experiment varied with the antigen/ antibody ratio when DC were used as accessory cells (Fig 6). Maximal

Figure 4

F(ab')₂ fragments of anti-OVA antibody were obtained by pepsin digestion of intact affinity purified antibody. Anti-OVA activity of the fragments was determined by ELISA before use in assays. 5×10^4 DC were cultured with 10^5 CD4+ T cells in the presence of OVA only, OVA and specific antibody at 30 µg/ml, or OVA and F(ab')₂ specific antibody at the same concentration (see key). The experiment was carried out with cells from two sheep. The results shown are the mean counts of triplicate cultures \pm SDs. The statistical significance of the difference between CD4+ T cell proliferation to OVA only, and the response on addition of antibody, was assessed using a Two Sample T Test.

FIGURE 4 T Cell Proliferation is Not Enhanced by
F(ab')₂ Portions of Specific Antibody

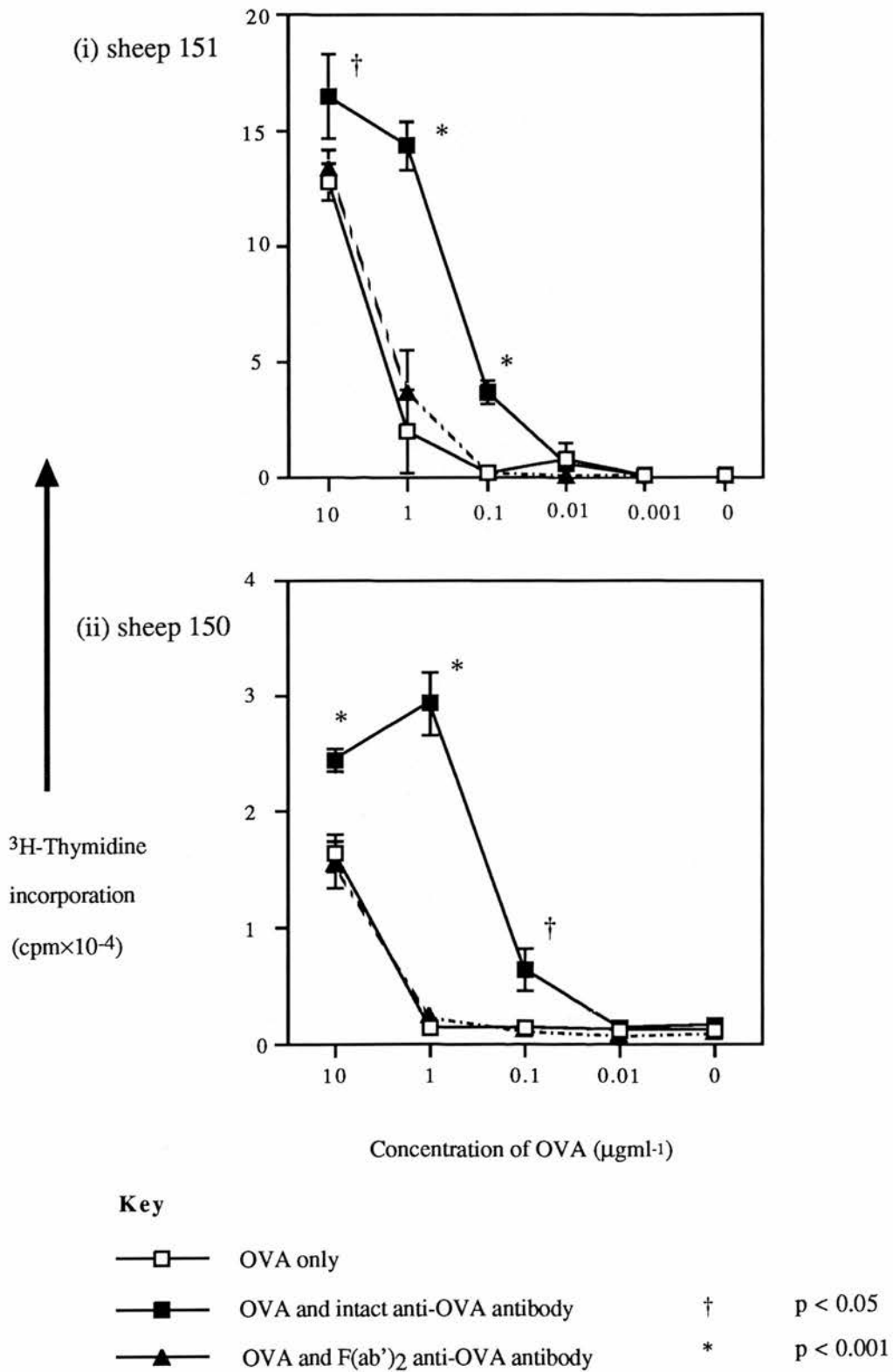


Figure 5

DC and CD4⁺ T cells were purified as described and cultured with OVA dilutions only or OVA plus specific antibody at 30 μ gml⁻¹. Anti-MHC class II monoclonal antibody, SW73.2, was added to some wells at 20 μ gml⁻¹, and was present throughout the five day incubation period. Rat anti-mouse IgG, used at the same concentration as SW73.2, served as a control. The results shown are the means of triplicate cultures \pm SDs. The statistical significance was assessed using a Two Sample T Test. The proliferation of T cells to OVA and anti-OVA antibody was compared with that to OVA only. The response to OVA, anti-OVA antibody and the control antibody was compared with the proliferation to OVA and anti-OVA antibody. T cell proliferation to OVA, anti-OVA antibody and SW73.2 was compared with the T cell response to OVA and anti-OVA antibody.

FIGURE 5 Effect of Anti-MHC Class II Antibody on
Enhanced T Cell Stimulation

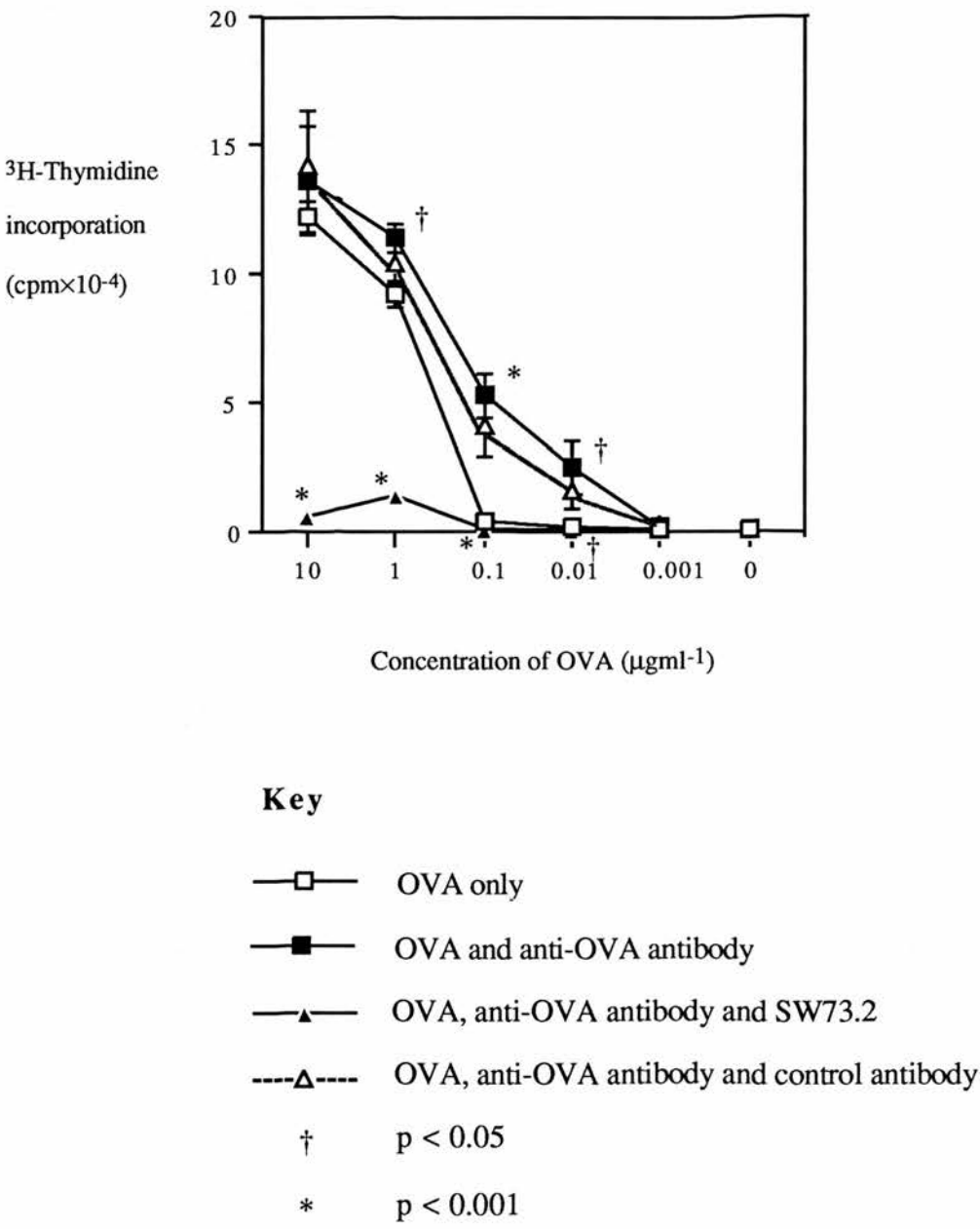
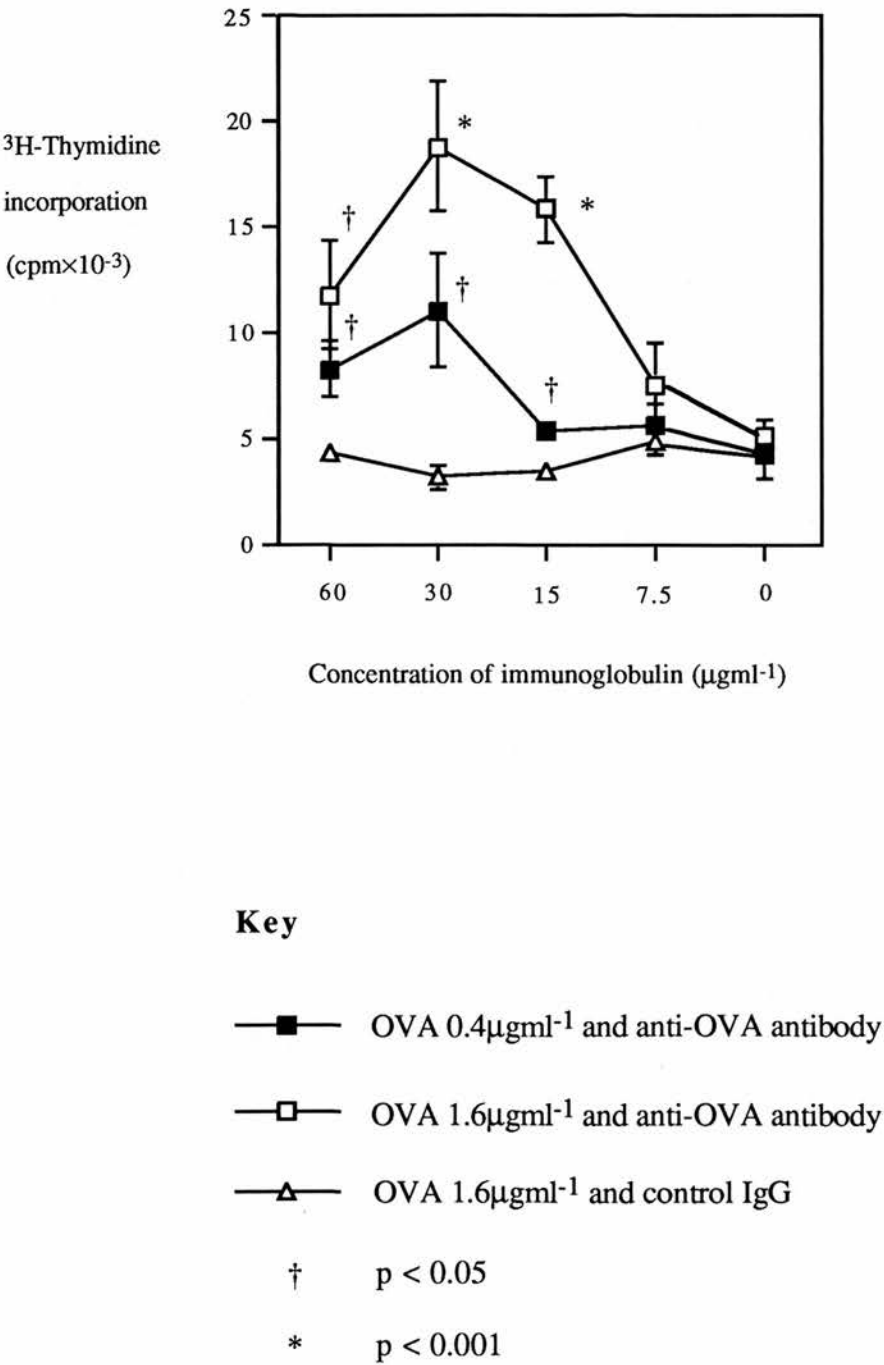


Figure 6

5×10^4 DC and 10^5 CD4+ T cells were plated with fixed suboptimal concentrations of OVA (1.6 or $0.4 \mu\text{gml}^{-1}$), with or without added specific antibody at a range of concentrations (see key). IgG from an unprimed sheep was used as a control over the same range in concentration. Proliferation of triplicate cultures was assessed by measurement of ^3H -thymidine incorporation over the last five hours of culture. Mean counts \pm standard deviations are illustrated, with p values calculated using a T Test as previously.

FIGURE 6 The Effect of Antibody/ Antigen Ratio
on Proliferation of CD4+ T Cells



enhancement of proliferation over that with OVA alone occurred at an antibody concentration of $30\mu\text{gml}^{-1}$, with OVA at 1.6 or $0.4\mu\text{gml}^{-1}$. These antibody and antigen concentrations represent a 1.9-fold and a 7.5-fold antibody excess respectively ($3\mu\text{g}$ of OVA would be required for equivalence with $30\mu\text{g}$ antibody). A smaller increase in proliferation occurred when the antibody concentration was increased or decreased from this level. The response of CD4+ T cells to OVA in the presence of IgG from an unprimed sheep is shown in the same figure. No additional proliferation above that to OVA alone occurred over the same concentration range of IgG.

Fig. 7 illustrates the results obtained in separate experiments with three sheep. Again the greatest increase in T cell proliferation on the addition of specific antibody is seen at moderate (30-fold) antibody excess (i), with highly significant 14-fold, 18.5-fold and 6.3-fold increases observed in sheep 150, 151 and 21 respectively. DC and T cells were cultured with antibody at a fixed concentration of $30\mu\text{gml}^{-1}$, with OVA concentrations of $0.1\mu\text{gml}^{-1}$ in (i), 10ngml^{-1} in (ii) and 1ngml^{-1} in (iii). The increase in proliferation with added specific antibody was significant in two of the three sheep in (ii), representing a 300-fold antibody excess, but was not significant in any of the sheep where OVA was used at 1ngml^{-1} (iii), which gave a 3000-fold antibody excess. Experiments involving the addition of specific antibody at antigen excess were carried out in three sheep; in one animal no increase in T cell proliferation was observed, while in two sheep significant enhancement in the T cell response occurred at a 3.3-fold antigen excess. However, higher concentrations of antigen were present than were used in the experiments at antibody excess, with OVA at $10\mu\text{gml}^{-1}$, and CD4+ T cells proliferated strongly to OVA alone (Fig.2 and data not shown).

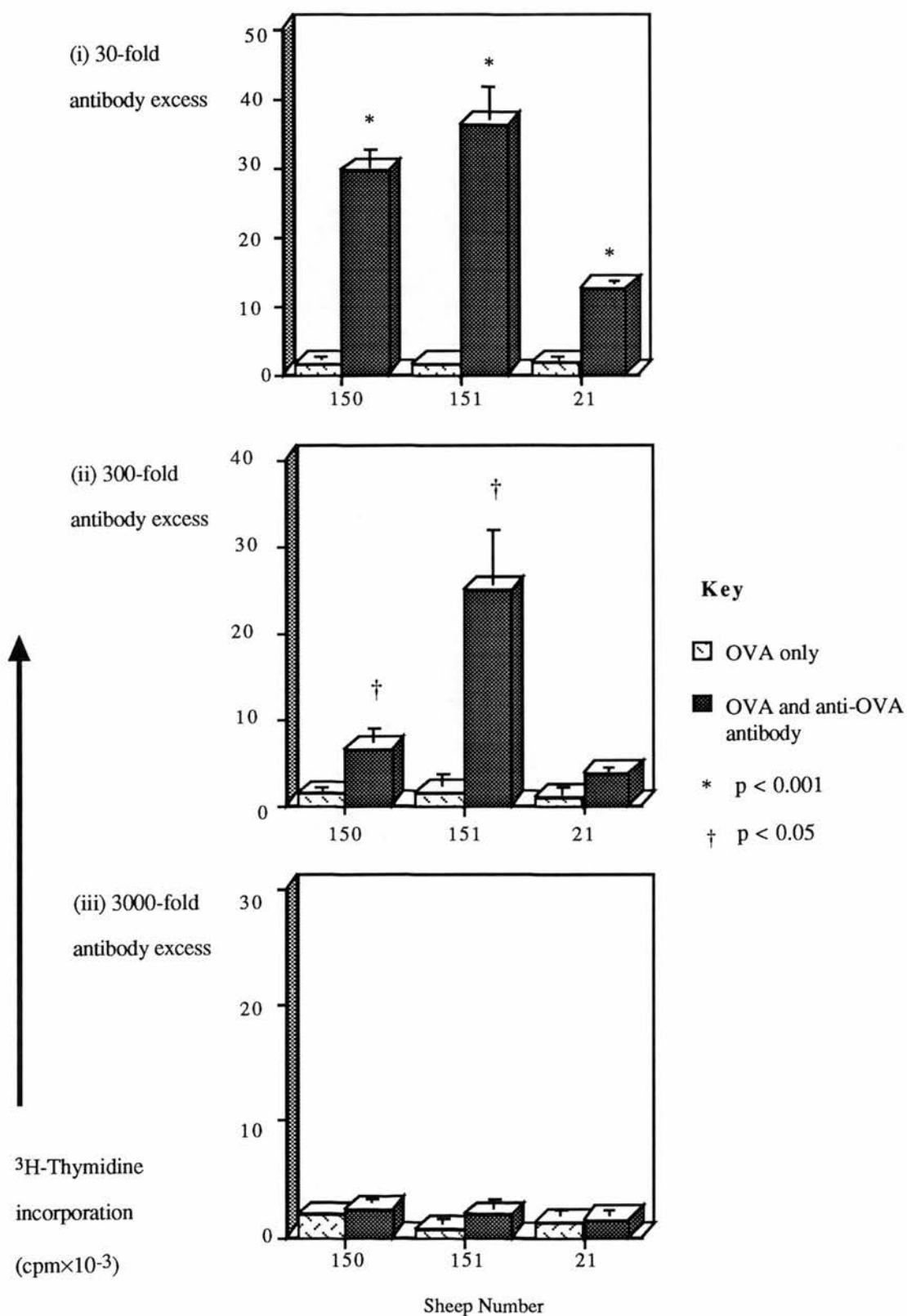
6.7. Effect of Altering DC Numbers on T Cell Proliferation

DC are potent accessory cells for the stimulation of T cells and are often effective in smaller numbers than other APC types. Experiments were carried out with fixed levels of OVA ($0.2\mu\text{gml}^{-1}$) and specific antibody ($30\mu\text{gml}^{-1}$), with a constant number of CD4+ T cells and varying numbers of afferent lymph DC.

Figure 7

DC and CD4⁺ T cells were cultured with OVA at suboptimal concentrations, or OVA and anti-OVA antibodies at 30 μ gml⁻¹. In (i), OVA concentration was 0.1 μ gml⁻¹, representing a 30-fold antibody excess; in (ii) OVA was used at 10ngml⁻¹, representing a 300-fold antibody excess, and in (iii) an OVA concentration of 1ngml⁻¹ gave a 3000-fold antibody excess. The results are means of triplicate cultures \pm SDs, and the statistical significance values for the differences between proliferation to OVA alone and response to OVA and antibody were determined using a Two Sample T Test.

FIGURE 7 Antibody/ Antigen Ratios: Effect on
T cell Proliferation in Three Sheep



As expected, the proliferation of T cells to OVA presented by DC decreased with the number of DC added (Fig. 8). The enhanced T cell response in the presence of anti-OVA antibody also decreased with DC number, but the difference between proliferation to OVA alone and that to OVA and antibody was greatest at the lower numbers of DC. Indeed at the smallest number of DC added, T cell proliferation to antigen alone was at background levels yet on addition of specific antibody a 400-fold increase occurred.

6.8. Discussion

The experiments in this chapter were carried out to investigate the effect of specific antibody on presentation of antigen by DC. The enhanced uptake of antigen in complex form by these cells has already been demonstrated; however the functional consequences with regard to effects on antigen presentation remain to be determined.

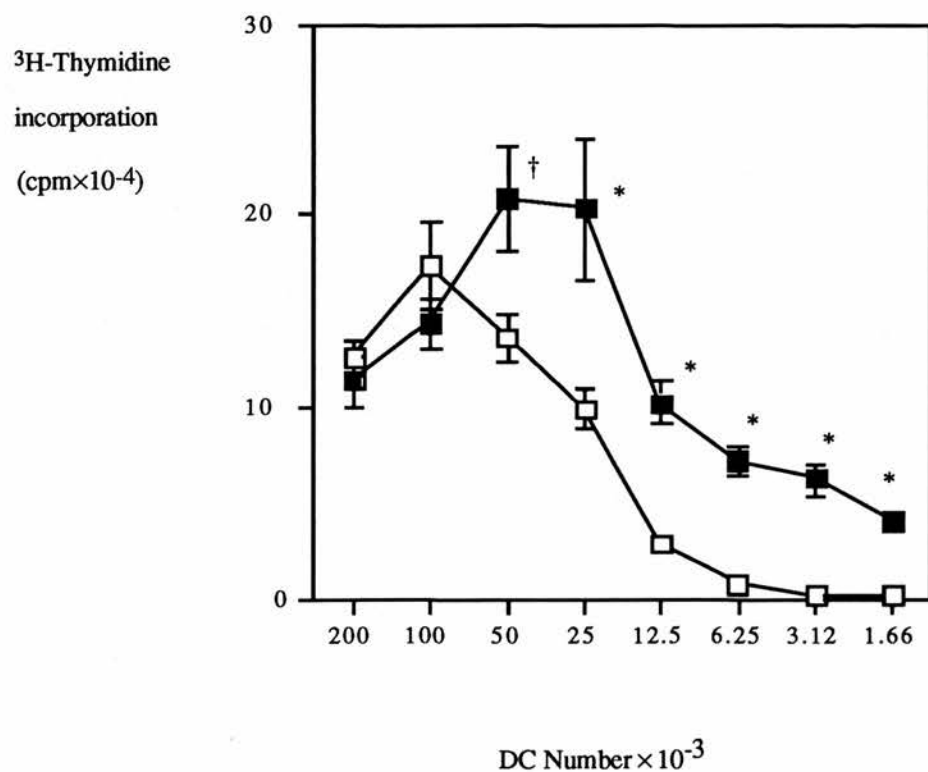
Proliferation of T cells was used as a measure of antigen presentation by DC, as there is as yet no direct method of assessing the immunostimulatory functions of APCs. A population of responding T cells free of APCs was required to investigate the presentation of antigen by DC. This was obtained by the positive selection of CD4⁺ T cells from PBMs using a MACS. Selected cells were analysed by FACS to ensure that purity was at least 93% before culture with OVA or OVA and specific antibodies in the absence of added APCs. CD4⁺ T cells showed no proliferation over that seen in medium only when plated with stimulatory or suboptimal concentrations of OVA, or OVA and specific antibodies, unless DC were added, indicating that the responding cells were not contaminated with APCs.

The cells obtained after centrifugation of afferent lymph cells over metrizamide were 60-80% DC by morphological criteria on cytopins, with some lymphocytes also present. Before use as APCs this cell population was irradiated with a dose of 2500 rads to prevent proliferation of lymphocytes and possible antigen presentation by B cells. After irradiation the APCs showed no proliferation above background levels when cultured with OVA or OVA plus antibody, thus the proliferative response observed in these assays was due to the CD4⁺ responder cells.

Figure 8

CD4⁺ T cells (10^5) were cultured with OVA at $0.2\mu\text{gml}^{-1}$, with and without specific antibody at $30\mu\text{gml}^{-1}$ and varying numbers of DC. The results are the means of triplicate cultures \pm SDs. Statistical analysis was carried out with a Two Sample T Test.

FIGURE 8 Enhanced CD4+ T Cell Stimulation
by Varying Numbers of DC



Key

- OVA only
- OVA and anti-OVA antibody
- \dagger $p < 0.05$
- * $p < 0.001$

DC and CD4⁺ T cells from primed sheep were cultured with OVA at a range of concentrations. At suboptimal levels of OVA, the addition of specific affinity purified antibodies greatly enhanced T cell proliferation. This highly significant increase was seen in experiments with six sheep. Although some enhancement was seen at higher levels of OVA, the potentiation of the T cell response was greatest at concentrations of OVA that caused little proliferation alone. The data suggests improved antigen presentation by DC following uptake of OVA in the form of immune complexes.

To confirm the requirement for an intact Fc portion of antibody to interact with DC surface Fc receptors, F(ab')₂ fragments of specific antibody were used in place of whole antibody molecules. No intact IgG remained in these preparations. F(ab')₂ portions of specific antibody were found to be ineffective in these assays; no increase in T cell proliferation occurred when digested antibody was used at the same concentration as intact antibody. This was not due to reduction or loss of anti-OVA activity during pepsin digestion, as F(ab')₂ preparations had similar titres to intact antibodies when tested by ELISA. An Fc portion of antibody is therefore required for the enhancement of proliferation seen in these experiments, implicating Fc receptors for IgG in the process.

To exclude any non-specific stimulatory effects of affinity purified antibodies, anti-HSA antibodies prepared from the serum of a primed sheep on an affinity column were used as a control in some experiments. These antibodies had anti-HSA activity when tested by ELISA, but had no stimulatory effect on T cell proliferation to OVA dilutions. This result confirmed the requirement for specific antibody capable of immune complex formation with OVA.

The degree of the increase in uptake of antigen by afferent lymph DC in the presence of specific antibody varied with the antigen/ antibody ratio (Harkiss et al 1990); experiments carried out in this chapter clearly show that the magnitude of the enhancement of T cell proliferation on addition of anti-OVA antibody also depends on this ratio. Maximum potentiation of the T cell response was seen when specific antibody was present at moderate excess, while in extreme antibody excess or antigen

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excess smaller increases in proliferation occurred for the same OVA concentration. Moderate antibody excess therefore provides the optimum conditions for the enhanced presentation of antigen by DC in the presence of specific antibody. This could be accounted for by the uptake of complexes being most efficient at moderate antibody excess, or alternatively the processing and presentation pathway followed by the immune complexes may function more effectively in conditions of moderate antibody excess.

The results from the experiment including anti-MHC class II monoclonal antibody indicated that the increased stimulation of T cells on addition of specific antibody at suboptimal OVA concentrations was dependent on a class II presentation pathway. At least some of the antigen taken in via the Fc γ R on the DC surface is therefore routed to a class II processing pathway.

Very low numbers of DC were effective at inducing additional T cell proliferation in the presence of antibody. In the experiment described, 1.6×10^3 DC were sufficient to cause a 400-fold increase in response to OVA when anti-OVA antibody was added in moderate antibody excess. Indeed, greater increases were observed with the smaller numbers of DC, while when 2×10^5 DC were used as APCs T cells proliferated strongly to OVA alone and no increase was seen on addition of antibody. This may reflect the importance of limited numbers of antigen-carrying DC *in vivo* which will encounter and activate specific T cells in secondary responses.

The enhancement of T cell proliferation by specific antibody but not by F(ab')₂ fragments, and the effects of irrelevant antibody and anti-MHC class II antibody are similar to those observed with unfractionated PBMs (Chapter 5). However, the degree of increase in the proliferative response to antigen on the addition of specific antibody was much greater in the DC/ CD4⁺ T cell assays than in experiments with PBMs. In the PBM assays, the maximum enhancement was a 5.8-fold increase in proliferation, compared with a maximum 400-fold increase in CD4⁺ T cell proliferation to antigen presented by DC. DC are potent APCs for soluble protein antigens (Van Voorhis et al

1983, Steinman 1991), and they may also be more efficient than monocytes, likely to be the major APC population in the PBMs, in the uptake and presentation of antigen in the form of immune complexes. Another difference between the results obtained in the two assay systems related to the antibody/ antigen ratio. In the DC/ CD4+ T cell assays, maximum potentiation of the T cell response was observed at moderate antibody excess, while no significant increase in proliferation was seen at extreme antibody excess. In the PBM assays, although the greatest increase in proliferation also occurred in moderate antibody excess, a significant enhancement of proliferation was also seen in extreme antibody excess. This difference may be due to variations in the type and quantity of Fc γ R expressed on DC and APCs in the PBM population, and therefore the optimal antibody/ antigen ratio for immune complex uptake, or to alternative pathways of antigen processing and presentation utilised by different cell types. However, to compare the two types of assay directly, purified monocytes and CD4+ T cells as responders would be required in place of unfractionated PBMs.

CHAPTER SEVEN

GENERAL DISCUSSION

7. GENERAL DISCUSSION

7.1. The Role of DC in the Primary Immune Response

It is generally accepted that DC are essential accessory cells for induction of the primary immune response to antigen (Inaba et al 1990, Macatonia et al 1989, Steinman 1991). Direct priming of naive T cells *in situ* by antigen-bearing DC has been demonstrated, while other APCs such as B cells and macrophages appear to be incapable of eliciting a response (McKeever et al 1992, Inaba et al 1990, Liu & MacPherson 1993). Some murine studies have suggested that B cells are required for T cell priming. However, animals in these experiments were treated with anti- μ antibody from birth in order to delete B cells, and this treatment could also have affected DC viability or function (Janeway et al 1987, Kurt-Jones et al 1988). DC are also required to elicit a primary antibody response, probably for the activation of specific CD4⁺ T cells which can then provide B cell help (Inaba et al 1985). The reasons for the distinctive capabilities of DC have not been elucidated, although several features of these cells are probably important.

Interstitial DC, situated in non-lymphoid tissues such as the epidermis, are ideally situated to encounter and accumulate antigen rapidly on its entry into the body. The method by which DC take up antigen in a primary response is still unclear, although an efficient process of antigen accumulation is vital to ensure initiation of the immune response. Non-specific pinocytosis may account for the uptake of soluble antigens (Barfoot et al 1989), and a role has been suggested for IgM, detected on the surface of a variable proportion of DC from sheep afferent lymph (Bujdoso et al 1990). Specific IgM antibodies, generated during the primary response, may form complexes with antigen and attach to the DC surface via complement receptors (Steinman 1991, Nestlé et al 1993) or possibly Fc μ R. "Natural" antibodies of IgM isotype, with multiple specificities, could act as a concentrating mechanism for antigen before the production of specific antibodies. The role of cytophilic IgM in antigen uptake by afferent lymph DC in the primary immune response was investigated in Chapter 3. *In vitro* studies assessed DC uptake of FITC-OVA in the presence or absence of purified IgM anti-OVA

antibodies, or unfractionated serum containing these antibodies. No increase in DC uptake of labelled antigen was observed in the presence of either serum or purified antibodies. This may indicate that IgM does not function in the uptake of antigen by DC in the primary response *in vivo*; alternatively the *in vitro* experimental conditions could have been unsuitable. It is possible that a lack of complement is responsible for the lack of uptake; 7S subunits of IgM could also be involved, rather than the complete IgM pentamer. Further experiments would be required to investigate these possibilities.

Although DC are capable of internalising soluble antigens, they are generally thought to be poorly phagocytic (Steinman 1991), and this raises the question of how a primary response to particulate antigens and microorganisms is initiated. However, other reports describe the presence of phagocytic inclusions in afferent lymph DC, and the uptake of *Bacillus Calmette Guérin* (BCG) organisms by proliferating DC precursors from murine bone marrow (Hall & Robertson 1984, Inaba et al 1993). DC pulsed *in vitro* with BCG elicited strong T cell responses *in vivo*, demonstrating that at least at this stage of differentiation, DC are capable of taking up microorganisms and processing antigens for presentation in immunogenic form to naive T cells.

After taking up antigen, DC migrate via the afferent lymph or the blood to the draining lymph node or spleen, where they interact with recirculating T cells. Naive T cells recirculate through the lymph node, passing from high endothelial venules to T cell areas of the node and subsequently into the efferent lymph (Mackay et al 1990). Memory-type T cells, in contrast, tend to migrate through the peripheral tissues and enter afferent lymph (Pitzalis et al 1988). Activated antigen-specific T cells secrete cytokines necessary for providing B cell help in antibody production, and for promoting cell-mediated responses.

The superior accessory ability of DC compared with other APC types is unlikely to be due to differences in the method or efficiency of antigen uptake. Macrophages, for example, are far more efficient in the phagocytosis of particulate antigens than DC, and also accumulate soluble antigens by fluid phase endocytosis. However, macrophages and monocytes are incapable of activating naive CD4⁺ T cells (Inaba et al 1990, Levin

et al 1993). B cells acquire soluble antigens by pinocytosis. Specific antigen, recognised by the mIg of the B cell, is taken up and presented with great efficiency to T cell lines (Lanzavecchia 1985), but B cells are not able to activate naive T cells. It is probable that the pathways of processing and presentation and the costimulatory characteristics of the different APC types are more important.

Exogenous antigens are generally processed in an endocytic pathway for presentation by MHC class II molecules, while cytoplasmic antigens are processed via a separate route and presented in association with MHC class I molecules (Harding 1991). The class II pathway requires the presence of acidic intracellular compartments and is blocked by lysosomotropic agents such as chloroquine. There may be differences in the pathway or in the proteolytic enzymes in DC as compared to other APCs such as monocytes, enabling DC to produce antigenic peptides for association with MHC class II molecules more efficiently.

Although DC constitutively express higher levels of MHC class II molecules than other APCs, this alone is unlikely to account for their unique abilities: no correlation was found between the level of class II expression by different APC types and their ability to prime naive CD4⁺ T cells *in vivo* (Levin et al 1993). However, there may be qualitative differences in DC class II molecules or in the array of peptides generated by DC for association with class II, which increase affinity for the TCR of antigen-specific T cells. DC have fewer lysosomal enzymes than monocytes (Thomas et al 1993a), and it is therefore possible that more peptides escape complete degradation and remain available for interaction with the antigen binding groove of MHC class II molecules. Monocytes and DC were found to have comparable accessory activity in assay systems such as those involving anti-CD3 mAb stimulation of T cells, which depend less on the expression of MHC class II by the APC. Costimulatory molecules were employed comparably by each cell type in these assays. However, in the MLR, which is dependent on class II expression, DC were far more effective than monocytes in the activation of naive and memory T cells, suggesting that differences in the expression of class II molecules and/or their peptide ligands are likely to be responsible for the superior ability of DC to activate allogeneic T cells (Thomas et al 1993b). This may be

important in both primary and secondary responses.

Ligation of the TCR by specific peptide on MHC molecules is necessary but not sufficient for T cell activation. Costimulation, mediated either by a surface molecule on an APC interacting with its ligand on the responding cell, or by cytokines produced by the APC, is required for activation. Lack of a sufficient costimulatory signal can lead to anergy. The ability of the accessory molecules ICAM-1, LFA-3 and B7 to provide costimulation through their T cell ligands has been described (Dustin & Springer 1989, Springer 1990, Young et al 1992). These molecules are present on monocytes and DC, and the ligand pairs seem to work in an additive manner for optimal T cell activation (Thomas et al 1993b). DC from ovine afferent lymph also express CD2 which may contribute an activational stimulus by interaction with LFA-3 on responding cells. B7 has been suggested as the central costimulatory molecule in the activation of responding cells by DC, due to their constitutive expression of this molecule, while it is found on other APCs only after activation (Young et al 1992). However, more recent studies have indicated that DC, like other APCs, acquire B7 expression and function on activation (Thomas et al 1993b, Hart et al 1993). B7 alone is therefore unlikely to account for the ability of DC to activate naive T cells and initiate a primary immune response.

Other characteristics of DC undoubtedly contribute to their accessory functions. Surface area is increased by the DC's dendritic morphology, enabling multiple interactions with T cells and the formation of clusters in which specific T cells are activated (Steinman 1991). DC surface molecules are sialated to a lesser degree than those of other cells, giving the cell membrane a lower negative charge and promoting DC association with negatively-charged responding cells (Boog et al 1989).

7.2. Accessory Activity of DC in the Secondary Immune Response

Although there is an absolute requirement for antigen presentation by DC for activation of naive T cells, this is not the case in the secondary response. APCs such as macrophages and B cells express MHC class II molecules and can act as accessory cells

for memory or activated CD4+ T cells (Unanue & Allen 1987). Other cell types can be induced to express class II molecules, although the consequences of class II expression by cells not specialised for antigen presentation are varied. While class II-bearing endothelial cells appear to be capable of T cell activation, antigen presentation by keratinocytes expressing MHC class II molecules leads to anergy (Geppert & Lipsky 1985, Bal et al 1990). T cells express MHC class II on activation (Yu et al 1980), and there is contradictory evidence concerning their possible role in antigen presentation. Activated T cells from ovine efferent lymph are effective stimulator cells in the MLR, but fail to present native protein antigens or peptides. In contrast, ovine T cell lines generated *in vitro* are capable of presenting peptides but not native antigens (Hopkins et al 1993). Activated human T cells can also stimulate responding cells in the MLR, and presentation of peptides to specific T cell responders has been reported (Damle & Gupta 1982, Gerrard et al 1985, La Salle et al 1991). The lack of ability to present native antigens may be due to deficiencies in uptake; when viral antigen binds to a T cell surface molecule uptake is enhanced and it can be effectively processed and presented (Lanzavecchia et al 1988).

Conflicting studies describe the induction of anergy, probably due to a lack of adequate costimulation, when antigen is presented by class II-expressing T cells in the absence of other APCs (Jenkins & Schwartz 1987, Sidhu et al 1992). Responding T cells could be activated where peptide was presented on T cell class II molecules, as long as costimulation was provided by added APCs (Sidhu et al 1992). The significance of antigen presentation by T cells *in vivo* is thus still unclear.

Other cell types can therefore act as APCs for activated or memory T cells. However, in many experimental systems used to assess antigen presentation, DC are the most efficient accessory cells in secondary responses (Van Voorhis et al 1983, McKeever et al 1991). The factors discussed above (section 7.1.) involving processing and presentation, and possible differences in DC expression of MHC class II and the peptides produced by DC as opposed to other APCs, could also contribute to DC accessory functions in the secondary response.

7.2.1. The Role of Fc γ R in Uptake and Presentation of Antigen by DC

The major differences in a secondary as compared to a primary response *in vivo* are the presence of memory T and B cells and specific antibody. The latter enables cells bearing FcR to accumulate and present antigen in complex form with greatly increased efficiency (Lanzavecchia 1990). The expression of Fc γ R by DC from different tissues and those obtained by separate methods of isolation is variable (Section 1.6.). There is evidence that DC from ovine afferent lymph carry Fc γ R; these cells formed rosettes with IgG-coated erythrocytes and took up fluoresceinated antigen with increased efficiency in the presence of specific antibody both *in vitro* and *in vivo* (Harkiss et al 1990, and Chapter 3). The enhancement was dependent on intact Fc portions of antibody, with F(ab')₂ fragments being completely ineffective, and the degree of increase in the uptake of antigen varied with the antibody/ antigen ratio. The data strongly suggests the involvement of Fc γ R on the DC surface in the accumulation of immune complexes. In accordance with this, an increase in surface IgG levels occurred when DC were incubated with OVA and anti-OVA antibody of IgG isotype (Chapter 3). This increase in staining was also dependent on the antibody/ antigen ratio, and can be explained by the presence of anti-OVA antibodies in the form of immune complexes attached to Fc γ R on the DC. The IgG detected on afferent lymph DC during these experiments was of the IgG1 isotype; no corresponding increase in IgG2 was observed. Harkiss et al (1990) reported that anti-OVA antibodies of both IgG1 and IgG2 isotypes were effective in promoting the uptake of OVA; however, the affinity purified anti-OVA antibodies used in this thesis were all IgG1, as determined by ELISA.

DC stained positively by flow cytometry with rabbit antisera raised against peptides from the sequence of bovine Fc γ RI, and an antiserum raised against a putative ovine Fc γ RII (Chapter 4). Although this clearly suggested the presence of Fc γ R on afferent lymph DC, it did not distinguish between the various types of Fc γ R. Western blot analysis of DC lysates with the anti-FP1 antiserum gave two bands at molecular weights of 40kD and 72kD, which were of the correct sizes for Fc γ RII and Fc γ RI as

described in mice and humans (Ravetch & Kinet 1991). Western blot analysis of ovine alveolar macrophage lysate with this antiserum also demonstrated two proteins of 40 and 72kD. Affinity purified anti-FP1 antibodies reacted with the same two bands, on lysates of both DC and alveolar macrophages. There is therefore considerable evidence for the expression of Fc γ R by ovine afferent lymph DC, and these cells appear to express both Fc γ RI and Fc γ RII.

Evidence for enhanced antigen presentation by DC following the uptake of complexed antigen via Fc γ R was obtained in the assays described in Chapter 6. This effect was observed in experiments with DC and CD4+ T cells from six sheep, with the degree of enhancement on the addition of antibody ranging from a 5-fold to a 302-fold increase. As with the uptake experiments, the enhancement was dependent on the antigen/antibody ratio, being greatest at moderate antibody excess, and required intact specific antibody. A significant increase in T cell proliferation was also observed at a 3-fold antigen excess in two of three sheep; however OVA was used at a higher concentration than in the antibody excess assays and there was a strong response to OVA alone.

Similar results were obtained in functional assays with PBMs, described in Chapter 5. Again, specific antibody caused a significant increase in the proliferation of PBMs from a primed sheep to low concentrations of OVA. However, the enhancement of the response was less than that seen with the DC and CD4+ T cell assays, ranging from a 1.8-fold to a 5.8-fold increase. The smaller increase may be due to the presence of fewer FcR-expressing APCs in the PBM experiments, or to the different APCs involved. DC may be more efficient in antigen presentation after uptake via FcR than monocytes. Further experiments with separated monocytes would be required to determine the explanation for this difference. F(ab')₂ portions of specific antibody had no effect on PBM proliferation, indicating the requirement for an intact Fc region of the IgG anti-OVA antibodies. The antibody effect also depended on the antibody/ antigen ratio, and irrelevant antibodies had no enhancing effect on PBM proliferation to OVA.

Enhanced presentation of antigen in the form of immune complexes has been reported for various FcR-expressing APCs (Celis & Chang 1984, Schalke et al 1985, Manca et

al 1991). DC from rat lymph node and thymus were effective at inducing enhanced proliferation of T cell lines to the acetylcholine receptor from *Torpedo californica*, in the presence of monoclonal antibodies specific for this antigen. Pulsing experiments with antigen and antibodies showed that the effect was at the level of the presenting cells, and DC incubated with complexes for one hour at 37°C were capable of eliciting a significant proliferative response from the responding cells. The authors concluded that a means for the attachment of IgG antibodies in complex form to the DC surface was involved, although rat splenic and thymic DC were not generally thought to express FcγR. This work, using rat cells, is the only previous report of enhanced antigen presentation by DC where the antigen is in the form of immune complexes. However, the assays used do not compare directly with the experiments described in Chapter 6, as monoclonal antibodies and T cell lines were used in the work by Schalke et al (1985), while polyclonal antibodies and a bulk population of CD4+ T cells from a primed animal were used in this thesis. The latter may more closely represent the situation *in vivo*, where antibodies recognising different epitopes of an antigen will be present. The DC were also obtained from different tissues in each case; from the spleen or thymus of the rat, and from the afferent lymph of sheep.

Polyclonal antibodies have been used by others to demonstrate the enhanced proliferation of human T cell clones to hepatitis B surface antigen in the presence of specific antibodies (Celis & Chang 1984). The CD4+ T cells used in the assays described in Chapter 6 had been recently isolated from peripheral blood, and not maintained for long periods in culture as is the case for T cell lines.

The importance of the antigen/ antibody ratio in the enhanced presentation of antigen in the form of immune complexes has been described (Manca et al 1991). In this study, the FcγR-expressing APCs were macrophages, and the responding cells were murine or human T cell clones specific for various antigens. Immune complexes in moderate antibody excess provided optimal stimulation of T cells, while complexes in extreme antibody excess or extreme antigen excess did not potentiate the T cell response to antigen alone. In conditions of extreme antibody excess, the proliferation of T cells to stimulatory antigen concentrations was actually inhibited. Antigen molecules were

shielded from degradation within the APC by the presence of many antibody molecules. This mechanism of shielding by antibody can alter the substrate available for processing, and may therefore influence which T cell epitopes are dominant in the response to a particular antigen (Watts & Lanzavecchia 1993).

The DC assays described in Chapter 6 gave similar results in that the maximum increase in the CD4⁺ T cell responses to suboptimal levels of OVA occurred at moderate antibody excess. However, specific antibody did not inhibit the T cell proliferation to OVA at any of the concentrations used. In the PBM assays described in Chapter 6, conditions of moderate antibody excess again provided optimal stimulation of the responding cells. In contrast to the DC and CD4⁺ T cell assays, and to the work carried out by Manca et al (1991), PBM proliferation to OVA was also enhanced at extreme antibody excess, although to a lesser degree than the increase observed at moderate antibody excess. The reason for this is unclear; it may be due to the different APCs involved in these experiments; DC and the APCs present in PBMs, likely to be monocytes. The latter may be more efficient in the uptake, processing and presentation of immune complexes at extreme antibody excess than DC from afferent lymph.

The antigen/ antibody ratio may be significant in the regulation of cell mediated and humoral aspects of the secondary response *in vivo*. A varying ratio in different tissues and at different stages in the secondary immune response, may mean that complexed antigen is present in either stimulatory or non-stimulatory form for presentation to specific T cells. Thus T cells would be efficiently activated to undergo clonal expansion and secrete cytokines, in a secondary response where residual antibody complexes with substimulatory concentrations of antigen for efficient uptake by Fc γ R-expressing APC. However, when levels of antibody had increased to give extreme antibody excess, the same APCs would not be able to stimulate specific T cells and the cell mediated response would decline. Antigen presentation by specific B cells is several orders of magnitude more efficient than that by B cells of irrelevant specificity (Lanzavecchia 1987). However, the presence of specific antibodies at extreme excess *in vivo* would leave few B cell epitopes of the antigen exposed for interaction with mIg and so the antibody response would be decreased. Cross-linking of Fc γ RII and mIg on B cells

delivers an inhibitory signal to the cell (Phillips & Parker 1984)

Fc γ R are internalised after immune complex binding, and enter an endocytic compartment from which both receptor and ligand proceed to lysosomes for degradation (Ukkonen et al 1986). In the DC assays, at least a proportion of antigen was subsequently presented on MHC class II molecules, and activated specific CD4+ T cells. Exogenous antigen is generally processed by the endocytic pathway before being presented in association with class II molecules, but antigens in the form of SMAA complexes and ISCOMs may also be presented on class I molecules to CD8+ T cells for initiation of CTL responses (De Vries et al 1988, Takahashi et al 1990, Randall & Young 1991). Presentation of viral protein incorporated into ISCOMs by a class II pathway that is not inhibited by chloroquine has also been demonstrated (Van Binnendijk et al 1992). In the latter case, ISCOMs are likely to enter the cytoplasm of the cell where antigenic peptides are produced, before association of these peptides with class II molecules either in the ER or after entering an endosomal/ lysosomal compartment in which class II molecules reside. The processing pathway responsible for presentation of OVA peptides on MHC class II molecules after DC uptake of antigen in the form of immune complexes was not determined in this thesis; however, as Fc γ R enter endosomes on internalisation, it is probable that complexes are processed by the chloroquine-sensitive endosomal pathway. It is possible that some of the antigen taken up by DC in complex form also enters the class I pathway, and is capable of eliciting cytotoxic responses from CD8+ T cells. Further experiments involving DC and purified CD8+ T cells would be required to determine whether this occurs.

Cross-linkage of Fc γ R causes internalisation, and delivers a signal to the cell. This signal may be inhibitory as in the case of the cross-linked B cell mIg and Fc γ R, or stimulatory as for Fc γ R on macrophages, monocytes and neutrophils (Van de Winkel & Capel 1993, Phillips & Parker 1984). Cross-linking Fc γ RIII on NK cells initiates cell activation and ADCC effector function. Initial signalling depends on tyrosine phosphorylation of the γ or ζ associated subunit of the FcR. This is rapidly followed by phospholipase C (PLC) activity, and hydrolysis of membrane inositol triphosphate

and diacylglycerol which mediate the mobilisation of intracellular calcium and the activation of protein kinase C (Ting et al 1992, Azzoni et al 1992). PLC-derived second messengers appear to be crucial for the development of ADCC function (Trinchieri 1989), but the protein tyrosine kinase linking FcR stimulation to PLC activation has not been characterised. Although the γ and ζ subunits situated with Fc γ RIII do not possess intrinsic enzymatic activity, they are associated with a 70kD tyrosine phosphoprotein. Stimulation of Fc γ RIII induces increased association of this phosphoprotein with the ζ subunit in T and NK cells (Vivier et al 1993). A similar protein located with the ζ subunit in T cells has been identified as a tyrosine kinase, and the FcR-associated dimers may interact with this non-receptor tyrosine kinase to initiate signalling and cell activation.

There is less information on signalling events following the cross-linkage of Fc γ RII. A recent study demonstrated the requirement for tyrosine phosphorylation for the internalisation of Fc γ RII and Fc γ RI in a human monocytic cell line (Ghazizadeh & Fleit 1994). Fc γ RII is not generally thought to be associated with any subunits in the cell membrane, which would imply that tyrosine phosphorylation of the cytoplasmic tail of the receptor occurs on stimulation. However, Fc γ RI, Fc γ RII and Fc γ RIII on human myeloid cells all appear to be located with γ - γ homodimers. 20% of the Fc γ RII on cultured human monocytes is associated with these subunits (Masuda & Roos 1993). Signalling may therefore be initiated in a similar way to that described above for Fc γ RIII.

The events which follow the cross-linking of Fc γ RII on afferent lymph DC by immune complexes have not been determined, and it is not known whether Fc γ RII on DC has associated subunits. It is likely, however, that tyrosine phosphorylation of either the cytoplasmic region of the receptor, or perhaps of an associated γ chain, initiates cell signalling and activation in a similar manner to that seen with Fc γ RIII on NK and T cells. Modulation of DC surface molecules, migration to T cell areas in lymphoid tissues and increased antigen processing and presentation to specific T cells could all be enhanced by cell activation following FcR ligation. Signalling via Fc γ R on neutrophils

and macrophages induces the release of inflammatory mediators such as leukotrienes and prostaglandins, and the inflammatory cytokines IL-1, IL-6 and TNF- α (Debets et al 1990, Krutmann et al 1990, Chantry et al 1989). DC may respond in a similar manner and produce costimulatory cytokines such as IL-1 for enhanced activation of specific T cells, although DC production of cytokines is still an area of some controversy (Section 1.5.).

DC may also play a regulatory role in immune responses, by producing soluble forms of Fc γ R. Soluble Fc γ RII is produced by human LC (Astier et al 1994), and could also be released by afferent lymph DC. Murine IgG binding factors inhibit *in vitro* production of antibody in a secondary response, and the soluble Fc γ RII released by human LC competes with membrane-associated Fc γ RII for the binding of immune complexes (Fridman et al 1992, Astier et al 1994). Thus soluble Fc γ RII could inhibit the uptake of immune complexes by macrophages and DC *in vivo*, which in turn would lead to less efficient presentation of antigen by these cells and downregulation of antibody and T cell responses.

7.3. Modulation of DC Phenotype in a Secondary Immune Response

The changes in DC phenotype detected by FACS analysis after administration of OVA to a primed sheep were rapid and comprehensive, affecting all of the surface molecules examined to some extent. Both the number of DC positive for a specific molecule, and the level of expression on the positive cells, were decreased. This could be due to downregulation of surface antigens on the same population of DC that is present in resting lymph, or to a discrete cell population with different characteristics entering the afferent lymph from the tissues. Similar changes in DC phenotype were observed after *in vitro* culture. Although surface markers were downregulated where DC were cultured with medium only or with OVA, a more pronounced decrease occurred in the presence of OVA and specific antibody. This suggests that in the *in vivo* experiment the same population of DC may be altering in phenotype, rather than a new population of cells entering the afferent lymph.

The cross-linking of Fc γ R on the DC surface by immune complexes and subsequent signalling may provide the stimulus for the initial downregulation of surface markers. After an initial decrease, the number of DC staining with mAbs for particular molecules returned to around the resting percentage positive after a variable time period. Indeed, with some molecules such as CD1 and MHC class II, increased expression was observed 24 hours after antigen administration compared with that at the start of the experiment. This is in agreement with previous work, in which the number of MHC class II molecules expressed per DC increased substantially, to twice the original level two days after antigen administration, and six times the starting level by day 5 following OVA injection in a primed sheep (Hopkins et al 1989). The increased expression of class II correlated with enhanced ability of DC to present a second antigen to specific T cells. The initial downregulation of MHC class II may be a mechanism to turnover those molecules on the DC surface already containing peptides, so that newly synthesised class II molecules can interact with peptides from the recently acquired antigen (i.e. OVA) for subsequent presentation to T cells. CD1 levels seem to alter in a similar manner after antigen administration *in vivo* and *in vitro*. Although the role of CD1 has not been fully elucidated, CD1 molecules have been reported to function in the presentation of microbial antigens or hsp to T cells bearing $\gamma\delta$ TCRs (Porcelli et al 1989, Haas et al 1993), and also to $\alpha\beta$ T cells which do not express CD4 or CD8 (Porcelli et al 1992). Why CD1 expression should be modulated after administration of OVA is unclear, although this could be a non-specific consequence of antigen uptake via Fc γ R.

Fc γ R with bound ligands are sorted to lysosomes for degradation, therefore the uptake of immune complexes by DC should lead to an initial downregulation of receptors on the cell surface. A subsequent increase could occur following cell activation, to enable the cell to take up more antigen in complex form. Alternatively the levels of Fc γ R on the DC from afferent lymph may be further decreased, as occurs with isolated LC on culture (Witmer-Pack et al. 1988). DC are capable of retaining antigen in immunogenic form for a period of up to 2 days in culture (Puré et al 1990); once sufficient antigen has been obtained downregulation of Fc γ R could decrease the likelihood of the first

antigen being displaced by another. In the *in vivo* experiments involving OVA administration to primed sheep, the percentage of DC staining with the anti-FP1 serum increased four-fold at the 4 hour time point in one sheep, and two-fold by 24 hours after antigen administration in another. These increases may reflect an upregulation of Fc γ R on the DC surface after antigen injection; however, the antiserum was not absorbed against OVA, and rabbit anti-OVA antibodies could also be binding to OVA exposed on the DC membrane. To determine the contribution of the rabbit anti-OVA antibodies, the experiment could be repeated using rabbit antiserum from which anti-OVA antibody activity had been removed. A further limitation with the antiserum is that it appears to react with both Fc γ RI and Fc γ RII on the DC surface, as described in the Western blot analysis in Chapter 4. Monoclonal antibodies specific for each ovine Fc γ R type would be required to fully investigate changes in Fc γ R expression after immune complex stimulation.

The mechanisms responsible for the alteration in the proportions of DC expressing the various surface molecules investigated are unclear. MHC class II molecules recycle from the cell membrane, through an endocytic compartment and back to the membrane, and this has been proposed as a means of loading surface class II dimers with fresh peptides (Reid & Watts 1990). However, only newly synthesised class II molecules appear to associate with peptides, therefore the recycling of class II may not play a significant role in antigen presentation to CD4⁺ T cells (Davidson et al 1991). If class II molecules on DC are internalised in a similar way and not returned to the cell membrane, this process could account for the rapid decrease in expression of these molecules after immune complex administration. Adhesion molecules could also be internalised and not replaced at the cell surface: alternatively downregulation of both adhesion and presentational molecules may be achieved by enzyme cleavage.

Alteration in DC expression of adhesion molecules may be necessary to enable the cells to leave the dermis or epidermis. DC must have some method of remaining in the tissues, and this is likely to involve interactions both with resident stromal cells and the extracellular matrix. Some or all of these adhesive interactions probably must be broken to enable DC to migrate to and enter afferent lymphatics; a decreased association with

T cells in secondary responses, but they can also elicit CD8+ cytotoxic responses *in vivo* and *in vitro* (Macatonia et al 1989, Nair et al 1993, Takahashi et al 1993). Although exogenous antigens such as OVA would generally be processed by the endocytic pathway for presentation on MHC class II molecules to CD4+ T cells, antigens administered in the form of SMAA complexes and ISCOMs elicit both CD4 and CD8 T cell responses. A proportion of the immune complexes containing OVA may be presented on class I molecules; this could be determined by experiments with DC and CD8+ T cells separated from PBMs. Inhibition experiments with Brefeldin A and chloroquine would distinguish between the class I pathway and the endocytic class II processing pathway. The types of cells activated by DC in a secondary response *in vivo* may be an important factor in determination of the outcome of the immune response to particular pathogens.

7.5 Final Conclusions

The main findings described in this thesis included the pronounced and rapid modulation of surface markers on DC obtained from the afferent lymph of primed sheep after administration of antigen. These alterations in DC phenotype may be required for efficient processing and presentation of antigen and migration to the T cell areas of the draining lymph node. Afferent lymph DC were found to express Fc γ RI and Fc γ RII, demonstrated by flow cytometry and Western blot analysis with antiserum raised against a peptide from the sequence of bovine Fc γ RI. Functional studies showed that specific antibody greatly increased the response of CD4+ T cells to substimulatory concentrations of antigen presented by DC, depending on the antigen/ antibody ratio. An intact Fc portion of specific antibody was required for potentiation of the T cell response. These results suggest enhanced presentation of antigen by DC following the uptake of immune complexes via Fc receptors. Similar observations were made in experiments with unfractionated PBMs. DC Fc γ R would play an important role in the uptake and presentation of antigen in secondary responses *in vivo*, in which specific antibody of IgG isotype is present.

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